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14. ABSTRACT

The p53 tumor suppressor is the most commonly mutated gene in human breast cancer. Upon genotoxic stress, p53, a sequence-specific transcription factor, induces target genes that mediate many cellular activities such as cell cycle arrest and apoptosis. While the mechanism by which p53 induces apoptosis is unclear, this pathway has rich potential as a target for cancer therapies. Thus, the purpose, of this proposal is to characterize the molecular basis of p53-mediated apoptosis. Throughout this funding period, I have found that the N-terminal transcriptional activation domain 1 (AD1) and the C-terminal basic domain (BD) of p53 is inhibitory, while activation domain 2 (AD2) is required for transactivation of IGFBP3 by p53. Interestingly, lack of AD1 and the BD is paralleled in nature, and the naturally occurring p53 isoforms activate the IGFBP3 promoter. Importantly, p53 containing the inhibitory functional domains can be activated to induce IGFBP3 when histone deacetylases (HDACs) are inhibited by HDAC inhibitors. Furthermore, I found that HDAC2 specifically inhibits p53 activity by inhibiting p53-DNA binding activity. Since histone deacetylase (HDAC) inhibitors are in clinical trials for cancer therapies, my results shed insight into how HDAC inhibitors may be used as breast cancer therapies.

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Breast Cancer, p53, apoptosis, Insulin-like Growth Factor Binding Protein 3 (IGFBP3), transcription, stable inducible cell lines

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INTRODUCTION

The p53 tumor suppressor is the most commonly mutated gene in human breast cancer. Upon genotoxic stress, p53 functions as a sequence-specific transcription factor that transactivates target genes that mediate a variety of cellular activities such as cell cycle arrest, apoptosis, DNA repair, and other p53-dependent activities. Although the mechanism by which p53 chooses to activate genes for apoptosis, rather than cell cycle arrest, is poorly understood, this p53-dependent apoptosis pathway has a rich potential as a target for breast cancer therapies. Thus, the purpose of this proposal is to characterize the molecular basis of p53-dependent apoptosis. The studies proposed and executed utilize MCF7 breast adenocarcinoma cells to study mechanisms which regulate the ability of p53 to transactivate a pro-apoptotic target gene IGFBP3 as well as other p53-induced and -repressed genes.

BODY

Task 1: Identify the amino acids or modifications within p53 basic domain residues 364-383 that facilitate transcriptional repression of the IGFBP3 promoter. **Research Accomplishments:** This task has been completed. I have found that the C-terminal basic domain is inhibitory for the activation of the IGFBP3 promoter by p53. In addition, I have found that the N-terminal activation domain 1 (AD1) of p53 is inhibitory for the induction of IGFBP3 (See Figure 3B in Appendix, Harms and Chen, 2005). Furthermore, I have found that the induction of IGFBP3 by p53 requires activation domain 2 (AD2), since deletion or mutation of AD2 renders p53 incapable of inducing IGFBP3 (See Figure 3C in Appendix, Harms and Chen, 2005). To understand the physiologic relevance of these findings, I analyzed the ability of p53 isoforms to induce IGFBP3. Importantly, I found that naturally occurring p53 isoforms which lack AD1 and the BD activate the IGFBP3 promoter (See Figure 4 in Appendix, Harms and Chen, 2005). **Thus, p53 functional domains AD1 and the BD are inhibitory for the induction of IGFBP3 while AD2 is required for the induction of IGFBP3.**

Task 2: Identify p53-associated proteins that facilitate p53 repression of the IGFBP3 promoter or that activate p53 to induce apoptosis in MCF7 cells. **Research Accomplishments:** This task has been completed. To further understand how the basic domain (BD) facilitated repression of IGFBP3, I generated a stable MCF7 cell line that inducibly expresses HA-tagged p53(ΔBD) has been generated (See Figure 7A in Appendix, Harms and Chen, 2005). Interestingly I found that p53(ΔBD) does not induce IGFBP3 mRNA (See Figure 7B in Appendix, Harms and Chen, 2005). Thus, deletion of the BD alone was not sufficient to induce IGFBP3 indicating that deletion of both AD1 and the BD are required for p53 to induce IGFBP3. Further investigation into mechanisms which facilitate the repression of IGFBP3 by p53 revealed that histone deacetylases (HDACs) were involved. Importantly, through the use of the HDAC inhibitor Trichostatin A, I have found that **histone deacetylases (HDACs) facilitate the p53 repression at the IGFBP3 promoter**. Specifically, I found that inhibition of HDACs with the HDAC inhibitor Trichostatin A enables full length endogenous and exogenous p53 to induce IGFBP3 mRNA (See Figure 8C &D in Appendix, Harms and Chen, 2005).

Task 3: Identify the regions within the IGFBP3 gene required for transcriptional regulation by p53. **Research Accomplishments:** This task has been completed. The IGFBP3 promoter contains eleven half-sites of the p53 responsive element (See Figure 2A in Appendix, Harms and Chen, 2005). **I have found that specific half-sites of the p53 responsive element within the IGFBP3 promoter are required** for the activation of the IGFBP3 promoter by p53 which lacks the inhibitory domains named p53(ΔAD1ΔBD) (See Figure 2 in Appendix, Harms and Chen, 2005). Specifically, deletion of four of the upstream half-sites abrogates the activation of the IGFBP3 promoter by p53(ΔAD1ΔBD) (See Figure 2E in Appendix, Harms and Chen, 2005). This was unexpected since seven half-sites remained. However, these data suggest that the unique organization of the half-sites is critical for the regulation of IGFBP3 by p53.

Task 4: Determine *in vivo* binding of p53, p53(ΔAD1ΔBD), and p53(ΔBD) to the IGFBP3 gene in MCF7 cells using the chromatin immunoprecipitation (ChIP) assay. **Research Accomplishments:** This task has been completed. Interestingly, although only p53(ΔAD1ΔBD) is competent to strongly induce IGFBP3 (See Figure 7 in Appendix, Harms and Chen, 2005), I found that the **all p53 forms tested [p53, p53(ΔAD1ΔBD), and p53(ΔBD)] bind the IGFBP3 promoter by ChIP analysis** (See Figure 7 in Appendix, Harms and Chen, 2005). These data suggest that associated proteins or regulatory proteins act on p53 at the IGFBP3 promoter to regulate the ability of p53 to induce IGFBP3. In Task 2 I found that HDACs are proteins responsible for regulating the induction of IGFBP3 by p53.

Task 5: Characterize the role of IGFBP3 in p53(ΔAD1ΔBD)-mediated apoptosis. **Research Accomplishments:** This task has been completed. I have found that IGFBP3 plays an important role in p53(ΔAD1ΔBD)-mediated apoptosis (See Figure 6 in Appendix, Harms and Chen, 2005). Specifically, I found that p53(ΔAD1ΔBD)-mediated apoptosis was reduced when IGFBP3 was knocked down using siRNA targeting IGFBP3. In addition, I found that inhibition of IGFBP3 with a neutralizing antibody diminished the apoptosis induced by p53(ΔAD1ΔBD). Thus, **IGFBP3 is an important effector of p53(ΔAD1ΔBD)-mediated apoptosis**.

Task 6: Characterize the cooperation of p53 and IGFBP3 in the induction of apoptosis. **Research Accomplishments:** In task 5, I have found that IGFBP3 is an important effector of p53(ΔAD1ΔBD)-mediated apoptosis.

Additional work: In task 4, I found that both p53 and p53(ΔAD1ΔBD) bind the endogenous IGFBP3 promoter; however, only p53(ΔAD1ΔBD) induces IGFBP3. These data suggest that associated proteins regulate the ability of p53 to induce IGFBP3. In task 2, I found that histone deacetylases (HDACs) inhibit the ability of p53 to induce IGFBP3. Specifically, when HDACs are inhibited, endogenous and exogenous p53 becomes competent to induce IGFBP3. Importantly, HDAC inhibitors, which target many HDACs, are currently being tested as cancer therapeutics in phase I and II clinical trials. Thus, data generated from Tasks 2 and 4 led us to ask the mechanism by which HDACs negatively regulate p53. To this end, I used the MCF7 breast adenocarcinoma cell to generate individual cell lines which inducibly express siRNA targeting HDAC1 and HDAC2, named MCF7-si-HDAC1 or MCF7-si-HDAC2, respectively (See Figure 1A in Appendix, Harms and Chen, 2007). I found that knockdown of HDAC2 by siRNA inhibited MCF7 cell proliferation, in a manner which partly dependended on p53, and knockdown of HDAC2 also induced cellular senescence (See Figure 1B-F and Figure 2 in

Appendix, Harms and Chen, 2007). Importantly, I found that knockdown of HDAC2 enhanced p53-DNA binding activity as determined by ChIP analysis (See Figure 6 in Appendix, Harms and Chen, 2007). Upon knockdown of HDAC2, augmented p53-DNA binding activity led to enhanced transcriptional activation and enhanced transcriptional repression by p53 (See Figure 4 and Figure 5 in Appendix, Harms and Chen, 2007). Specifically the p53 target genes p21, Mdm2, FDXR, and DKK1 are induced to a greater extent by p53 upon knockdown of HDAC2 (See Figure 5 in Appendix, Harms and Chen, 2007). In addition, c-Myc, a p53 repressed gene, is repressed to a greater extent upon knockdown of HDAC2 (See Figure 4 in Appendix, Harms and Chen, 2007). Thus, as a result of finding that HDACs regulate the ability of p53 to regulate IGFBP3 (Task 2), I found that HDAC2 functions to regulate p53 activity by inhibiting p53-DNA binding activity. Importantly, these data shed insight into a mechanism by which HDAC inhibitors may function to positively regulate p53 activity in breast cancer therapies.

KEY RESEARCH ACCOMPLISHMENTS

- Determination that the 5' half-sites of the p53 responsive element within the IGFBP3 promoter are required for efficient activation of the IGFBP3 promoter by p53(ΔAD1ΔBD)
- Identification that a double point mutation in the p53 AD1 ((L22Q/W23S) renders p53(ΔBD) inactive at the IGFBP3 promoter
- Identification that a functional AD2 is required for the activation of the IGFBP3 promoter by p53(ΔBD) and p53(ΔAD1ΔBD)
- Identification that naturally occurring p53 isoforms that lack the inhibitory domains activate the IGFBP3 promoter
- Identification that p53 and p53(ΔAD1ΔBD) bind to the endogenous IGFBP3 promoter by ChIP analyses
- Identification that histone deacetylase activity inhibits the ability of full length p53 to induce IGFBP3 mRNA
- Determination that IGFBP3 is an important effector of p53(ΔAD1ΔBD)-mediated apoptosis
- Knockdown of HDAC2 inhibits MCF7 cell proliferation in a manner that partly depends upon p53
- Knockdown of HDAC2 induces cellular senescence in MCF7 cells
- Knockdown of HDAC1 or HDAC2 does not induce the stabilization of p53 in MCF7 cells

- Knockdown of HDAC2 results in the repression of c-Myc in a manner that partly depends on p53

- Knockdown of HDAC2 augments the induction of p53 target genes p21, Mdm2, FDXR, and DKK1 by p53

- Knockdown of HDAC2 augments p53-DNA binding activity

REPORTABLE OUTCOMES

Degree Earned

Ph.D. earned by Kelly L. Harms in the Department of Cell Biology at the University of Alabama at Birmingham, Spring 2007

Manuscripts

Harms, K.L., and Chen, X. 2005. The C terminus of p53 family proteins is a cell fate determinant. ***Mol. Cell. Bio.*** **25**: 2014-2030.

Scoumanne, A., **Harms, K.L.**, and Chen, X. 2005. Structural basis for gene activation by p53 family members. ***Cancer Biol. Ther.*** **4**: 1178-85.

Harms, K.L., and Chen, X. 2006. The functional domains in p53 family proteins exhibit both common and distinct properties. ***Cell Death and Differentiation.*** **13**: 890-897.

Harms, K.L., and Chen, X. 2006. p19ras brings a new twist to the regulation of p73 by Mdm2. ***Sci. STKE.*** **337**: pe24.

Harms, K.L., and Chen, X. 2007. Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. ***Cancer Research***, in Press.

Published Abstracts

Kelly L. Harms and Xinbin Chen. 2004. The basic domain of p53 mediates transcriptional repression of IGFBP3. American Association for Cancer Research 95th Annual Meeting Abstract # 2551. Vol 45.

Kelly L. Harms and Xinbin Chen. 2004. The C-terminus is the cell fate determinant of p53 family proteins. 19th Annual National MSTP Conference. Keystone, CO. Abstract # 25, page 15.

Kelly L. Harms and Xinbin Chen. 2004. The C-terminus regulates p53 function. 12th International p53 Workshop. Dunedin, New Zealand. Abstract #109, page 58.

Kelly L. Harms and Xinbin Chen. 2005. The C-terminus regulates p53 family function. Association for Cancer Research 96th Annual Meeting Abstract # 3649

Kelly L. Harms and Xinbin Chen. 2005. Era of Hope Department of Defense Breast Cancer Research Program Meeting. Abstract P27-10.

Kelly L. Harms and Xinbin Chen. 2006. Negative regulation of p53 function by histone deacetylase 2. 13th International p53 Workshop. NYC.

Presentations

Kelly L. Harms. 2004. The C-terminus regulates p53 function. 12th International p53 Workshop. Dunedin, New Zealand. 15 minute presentation.

Development of Cell Lines

Generation of an MCF7 breast adenocarcinoma cell line inducibly expressing HA-tagged p53(ΔBD) in the tetracycline-off system

Generation of parental MCF7 tetracycline-on cell line named MCF7-TR7

Generation of cell lines which inducibly express siRNA targeting histone deacetylase 2 (HDAC2), named MCF7-si-HDAC2 clones 10, B18, B38

Generation of cell line which inducibly expresses siRNA targeting HDAC1, named MCF7-si-HDAC1-7

Generation of cell line which inducibly expresses siRNA targeting HDAC2 and constitutively expresses siRNA targeting p53, named MCF7-si-HDAC2-stable si-p53 clone 13

CONCLUSION

p53 is a tumor suppressor gene. Depending on context, p53 regulates transcription of distinct but overlapping sets of genes which mediate different p53-dependent activities such as apoptosis or cell cycle arrest. Mechanisms that facilitate differential target gene regulation by p53 family proteins are complex. In these studies, I have found that HDACs play important roles in the regulation of p53 transcriptional activities. Specifically, HDACs facilitate differential target gene regulation by p53 isoforms, and HDAC2 modulates the DNA-binding activity of p53.

Through the study of the differential transcriptional regulation of IGFBP3 by p53, I found that the C terminus of p53 was inhibitory, such that C-terminally truncated p53 isoforms induced IGFBP3 whereas the full-length isoform did not. For p53, the N-terminal activation domain 1 was also inhibitory. The unique organization of eleven half-sites of the p53-responsive element within the IGFBP3 promoter was critical for the regulation of IGFBP3 by C-terminally truncated p53 family proteins. Although the C terminus did not affect the ability of p53 to bind the IGFBP3 promoter, the inhibitory function of the C terminus in p53 family proteins was linked to the activity of HDACs. Inhibition of HDAC activity with TSA restored the ability of endogenous and exogenous full-length p53 to induce IGFBP3. Since we showed that IGFBP3 is an important pro-apoptotic target gene, the data suggest that the induction of p53-mediated apoptosis is under stringent regulation by HDAC activity as well as by the expression of p53 isoforms.

Further investigation into the mechanism by which HDACs negatively regulate p53 activity revealed that HDAC2 modulates p53 transcriptional activities through the regulation of p53-DNA binding activity. First, I found that knockdown of HDAC2 induced cellular senescence and inhibited MCF7 cell proliferation in a dose-dependent manner. Importantly, I found that knockdown of HDAC2 induced the repression of c-Myc in both p53-dependent and -independent manners. Furthermore, knockdown of HDAC2 enhanced the induction of p21, Mdm2, ferrodoxin reductase (FDXR), and dickkopf-1 (DKK1) by p53. The enhancement of p53 trans-repression and trans-activation was due to an enhancement in p53-DNA binding activity and not alterations in p53 stability or post-translational modification(s). Thus, HDAC2 negatively regulates p53 transcriptional activities by affecting p53-DNA binding activity.

In sum, I have found that HDACs play important roles in regulating the transcriptional activity of full-length p53 family isoforms and that HDAC2 plays an important role in the regulation of p53-DNA binding activity.

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CURRICULUM VITAE

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EDUCATION

1996 - 2000	B.S., Vanderbilt University
2000 - present	Trainee in the Medical Scientist Training Program, University of Alabama at Birmingham

HONORS AND AWARDS

1998	Howard Hughes Medical Institute Fellowship through Vanderbilt University
1999	Summer Undergraduate Research Fellowship (SURF), Pfizer
2000	*Magna Cum Laude* with Honors in Molecular Biology, Vanderbilt University
2000	Outstanding Research in Molecular Biology, Vanderbilt University
2000	*Phi Beta Kappa*, Vanderbilt University
2004	First Place, poster presentation, UAB Department of Cell Biology Retreat
2006	First Place, poster presentation, UAB MSTP Annual Retreat
2006	First Place, poster presentation, UAB Department of Cell Biology Retreat

PROFESSIONAL EXPERIENCE

2001 - 2003	Student Committee for MSTP
2003 - present	Ad hoc reviewer for ***Molecular and Cellular Biology***, ***Oncogene, Nucleic Acids Research,*** and ***Genes and Development***
2004 - 2005	Department of Cell Biology Student Representative for Cellular and Molecular Biology Distinguished Scholars Seminar Series Committee
	May 25, 2005 hosted **Dr. Samuel Benchimol, Ph.D.**, Head, Division of Cellular and Molecular Biology, Ontario Cancer Institute, Professor, Department of Medical Biophysics at the University of

Toronto
Seminar Title: “The p53 tumor suppressor network regulates cell cycle progression and apoptosis”
November 2, 2005 hosted **Dr. Carol Prives, Ph.D.**, DA Costa Professor of Biological Sciences at Columbia University

TEACHING EXPERIENCE

Summer 2003	Mentoring summer high school Research Intern for UAB Community OutReach Development (CORD) Center Summer Science Institute
Spring 2004	IBS Core Curriculum, 2 hours
Spring 2005	IBS Core Curriculum, 2 hours
Fall 2005	IBS Core Curriculum, 2 hours
Spring 2006	Molecular Cell and Tissue Biology for UAB Medical Students, 1 hour

RESEARCH FUNDING

2004 - 2006 Department of Defense Breast Cancer Research Program

P.I.:	Kelly Harms
Grant No:	W81XWH-04-1-0349
Title:	“Mechanisms of p53-Mediated Apoptosis”
Fund:	$90,000 over 3 years

PUBLICATIONS

Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., **McNaughton, K**., and Cavener, D. 2002. The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. ***Mol. Cell. Bio.*** **22**: 3864-3874.

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Harms, K.L., and Chen, X. 2006. The functional domains in p53 family proteins exhibit both common and distinct properties. ***Cell Death and Differentiation.*** **13**: 890-897.

Harms, K.L., and Chen, X. 2006. p19ras brings a new twist to the regulation of p73 by Mdm2. ***Sci. STKE.*** **337**: pe24.

Harms, K.L., and Chen, X. 2006. Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. ***Cancer Research***, in Press.

PUBLISHED ABSTRACTS

McNaughton, K., and Chen, X. The basic domain of p53 mediates transcriptional repression of IGFBP3. 2003. AACR Abstract # 3312. Vol 44. page 659.

Harms, K., and Chen, X. The basic domain of p53 mediates transcriptional repression of IGFBP3. 2004. AACR Abstract # 2551. Vol 45.

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Harms, K., and Chen, X. The C-terminus regulates p53 family function. 2005. Era of Hope Department of Defense Breast Cancer Research Program Meeting. Abstract P27-10.

Harms, K., and Chen, X. 2006. Negative regulation of p53 function by histone deacetylase 2. 13th International p53 Workshop. NYC.

INVITED SEMINARS

McNaughton, K. "Beyond the B.S." HHMI Community of Scholars Program, Vanderbilt University, June 16, 2003. Invited by Stevenson Professor Ellen Fanning

Harms, K. "p53 and Friends." Cell Biology Departmental seminars for 1st and 2nd year MSTP students, UAB, September 29, 2003. Invited by Professor Etty Benveniste, Chair of the Department of Cell Biology

Harms, K., and Chen, X. "The C-terminus regulates p53 family function." 12th International p53 Workshop, Dunedin, New Zealand, November 8, 2004

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The C Terminus of p53 Family Proteins Is a Cell Fate Determinant

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The p53 tumor suppressor is the most commonly mutated gene in human cancers. The ability of p53 to induce cell cycle arrest, apoptosis, DNA repair, and other p53-dependent activities is well known; however, the mechanism by which p53 induces a specific activity over another is unclear. Here, we showed that stringent regulation of and by p53 family isoforms facilitates differential target gene expression and thus determines cell fate. Through the use of engineered deletion mutants, we found that activation domain 2 is required for induction of the proapoptotic target gene insulin-like growth factor binding protein 3 (IGFBP3) by p53 and that the basic domain inhibits induction of this gene by p53. Thus, for the first time we provide evidence that the basic domain of p53 is inhibitory in vivo as has been determined in vitro. We also showed that the in vivo inhibitory activity of the basic domain depends upon activation domain 1, such that combined deletion of activation domain 1 and the basic domain was required to alleviate the inhibition by the basic domain. Importantly, deletion of the inhibitory functional domains, namely N-terminal activation domain 1 and the C-terminal basic domain, is paralleled in nature. We found that the IGFBP3 promoter was activated by p53(ΔNΔBD), which mimics a naturally occurring N- and C-terminally truncated human p53 isoform, and by p53AS, a C-terminally truncated murine p53 isoform generated through alternative splicing, but not by full-length human or murine p53. In addition, we found that the C termini of p63 and p73 inhibit the induction of IGFBP3, such that C-terminally truncated p63 and p73 isoforms induce the expression of IGFBP3, whereas full-length ones cannot. We also demonstrated that IGFBP3 is an important effector of the apoptosis induced by N- and C-terminally truncated p53, such that knockdown of IGFBP3 by using an IGFBP3 neutralizing antibody or IGFBP3 small interfering RNA partially rescues the cell death induced by N- and C-terminally truncated p53. In addition, we identified that histone deacetylase activity, not p53 DNA binding ability, governs the regulation of IGFBP3 by full-length p53 family proteins, as inhibition of histone deacetylases restores the induction of IGFBP3 by exogenous full-length p53, p63, and p73 proteins. Furthermore, we found that activation of p53 or inhibition of histone deacetylases alone was not sufficient to induce IGFBP3; however, combined treatment endowed endogenous p53 with this activity. To better understand the significance of this regulation, we performed a microarray study and identified several target genes differentially regulated by full-length p53 and p53 lacking the N-terminal activation domain 1 and the C-terminal basic domain. Taken together, our data suggest a novel mechanism by which p53 family proteins differentially regulate gene expression and provide an insight for designing a combined therapy for cancer treatment.

The tumor suppressor p53 is the most commonly mutated gene in human cancers (43). After activation by cellular stresses, p53, a sequence-specific transcription factor, functions to transactivate genes that mediate cell cycle arrest, apoptosis, DNA repair, inhibition of angiogenesis and metastasis, and other p53-dependent activities (24, 30). The p53 protein contains several functional domains: activation domain 1 (AD1) within residues 1 to 42, activation domain 2 (AD2) within residues 43 to 63, the proline-rich domain (PRD) within residues 64 to 91, the sequence-specific DNA binding domain (DBD) within residues 100 to 300, the nuclear localization signal within residues 316 to 325, the tetramerization domain (TD) within residues 334 to 356, and the C-terminal basic domain (BD) within residues 364 to 393.

AD1 is important for transactivation; this domain contains residues that contact the basal transcriptional machinery (34). Previously, along with others we identified AD2 and characterized the requirement of AD2 for p53-dependent apoptosis (7, 8, 61, 70). In addition, along with others we have shown that the PRD is necessary for the induction of apoptosis and contributes to growth suppression (50, 60, 62, 67). The C-terminal BD has been subjected to extensive analysis. All evidence suggests that the BD is an important regulatory domain. Previous studies have shown that deletion of the BD and peptides or antibodies targeted to the BD increase p53-specific DNA binding activity in vitro (23, 25, 26, 55, 56). In addition, the function of the BD is altered through posttranslational modifications such as phosphorylation by casein kinase II or protein kinase C and acetylation by p300/CBP as well as through interactions with other proteins such as the calcium binding protein S100b and the DNA repair proteins XPB and XPD (reviewed in references 4 and 30).

Recently, the p63 and p73 proteins have been identified as p53 homologues (2, 28, 44, 59, 63). p53 family members share significant similarity at the amino acid level within the AD, the DBD, and the TD. Like p53, both p63 and p73 bind to the canonical p53-responsive element, transactivate p53 target gene expression, and induce apoptosis when overexpressed (reviewed in reference 64). Unlike p53, the genes encoding p63 and p73 are rarely mutated in human cancer (39, 65). Rather than displaying a propensity for tumor formation as in the p53 knockout mouse model (14), the p63 and p73 knockout animals demonstrate discrete developmental defects (39, 65). In addition, p63 and p73 undergo alternative splicing of their C termini, resulting in three p63 isoforms (α to γ) and seven p73 isoforms (α to η). These isoforms are transcribed from an upstream promoter as well as from a cryptic promoter within intron 3, called the TA and ΔN isoforms, respectively (reviewed in reference 64). Differential expression of these isoforms is believed to facilitate differential target gene regulation by p63 and p73.

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With the realization that each p63 and p73 isoform functions in similar yet different manners, new light is shed on the functional importance of previously identified p53 isoforms. Although different mechanisms generate p53 isoforms, mouse and human p53 exist as both N- and C-terminally truncated forms (reviewed in reference 11). N-terminal truncation of human and mouse p53 yields ΔNp53 and Δ40p53, respectively. Human ΔNp53 is generated through alternative splicing of intron 2 with the subsequent use of the translational start site at codon 40 (21). Mouse Δ40p53 lacks the first 40 amino acids and is generated through the use of an internal translational start site at codon 41. C-terminal truncation of human and mouse p53 yields I9+p53 and p53AS and occurs through alternative splicing of introns 9 and 10, respectively (1, 18, 32). In addition, several studies strongly suggest that calpain and interaction with mismatched DNA induce both N- and C-terminal cleavage of p53 (31, 37, 40, 42, 45). To date, the biological functions of these p53 isoforms remain unclear.

Insulin-like growth factor binding protein 3 (IGFBP3) belongs to the IGFBP family. There are six known members (reviewed in reference 17). Previously, IGFBP3 was shown to be regulated by p53 (5). IGFBP3 contains eleven decamers of the p53-responsive element within the promoter (3) and two canonical p53-responsive elements, called box A and box B, within introns 1 and 2, respectively (5). Since IGFBP3 is the major serum IGFBP, many IGF-dependent functions have been described; however, recent evidence supports the IGF-independent functions of IGFBP3. Interestingly, IGF-independent functions oppose the IGF-dependent functions, such that IGFBP3 directly inhibits growth, induces apoptosis, and sensitizes cells to apoptosis (6, 22, 29, 53).

Here, we showed that p53 isoforms differentially regulate target gene expression. Through the use of engineered deletion mutants, we first found that p53 functional domains dictate target gene specificity. We found that AD2 is required for, and the basic domain inhibits, induction of IGFBP3 by p53. Subsequently, we found a common theme among the p53 family of transcription factors: the C terminus of p53 family isoforms inhibits transactivation of IGFBP3, such that C-terminally truncated p53, p63, and p73 isoforms induce the expression of IGFBP3, whereas full-length ones cannot. We showed that IGFBP3 is an important mediator of p53(ΔAD1ΔBD)-dependent apoptosis. We also determined that histone deacetylase (HDAC) activity governs the induction of IGFBP3 by full-length p53 family proteins, as the inhibition of HDACs restores the ability of exogenous full-length p53, p63, and p73 isoforms to induce IGFBP3. Interestingly, we found that the simultaneous stabilization of p53 through the DNA damage pathway and the inhibition of HDACs restore the ability of endogenous full-length p53 to induce IGFBP3. Our data suggest a mechanism by which p53 family isoforms differentially interact with regulatory proteins to facilitate a highly regulated gene expression profile.

MATERIALS AND METHODS

Plasmids and mutagenesis. Human p53 proteins encoding full-length p53, p53(R249S), p53(ΔAD1ΔBD), p53(ΔAD1), p53(ΔBD), p53(AD1$^-$ΔBD), p53 (ΔAD2ΔBD), p53(AD2$^-$ΔBD), and p53(ΔAD1AD2$^-$ΔBD) were as previously described (69). All cDNAs, except p53(R249S) and p53(AD1$^-$ΔBD), were N-terminally hemagglutinin (HA)-tagged. To generate p53(ΔC20), a human cDNA fragment that encodes residues 1 to 373 was amplified by the 5′ end primer 5HA (GATCGAATTCACCATGGGCTACCCATACGATGTTCCAGATTACGCTGAGGAGCCGCAGTCAGATCC) and the 3′ end primer C373 (AGAATTCTCACTTTTTGGACTTCAG). To generate p53(ΔC10), a human cDNA fragment that encodes residues 1 to 383 was amplified by the 5′ end primer 5HA and the 3′ end primer C383 (AGAATTCTCAGAGTTTTTTATGGCG). To generate ΔNp53, a human cDNA fragment that encodes residues 40 to 393 was amplified by the 5′ end primer ΔNp53 (AGAATTACCATGGATGATTTGATGCTGTCCCCGG) and the 3′ end primer C393 (AGAATTCTCAGTCTGAGTCAGGCCCTTCTGTC). To generate p53(ΔNΔBD), the 3′ end cDNA fragment starting from the StuI site in ΔNp53 was replaced with the corresponding cDNA fragment in p53(ΔBD). To generate I9+p53, a human cDNA fragment that encodes an alternative splice form of p53 was generated by using the 5′ end primer 5hp53 (AGAATTCACCATGGAGGAGCCGCAGTCAGATCCTA) and the consecutive 3′ end primers I9-up (TTGAAAGCTGGTCTGGTCCTGAAGGGTGAAATATTC) and I9-dn (AGAATTCTTAACAATTTTCTTTTTGAAAGCTGGTCTGGTC). Full-length murine p53 was generated by using 5′ end primer 5mp53 (AGAATTCACCATGACTGCCATGGAGGAGTC) and the 3′ end primer mC387 (TGAATTCTCAGTCTGAGTCAGGCCCCAC). p53(A135V), a murine cDNA that encodes a naturally occurring p53 DNA binding mutant was as previously described (38). To generate Δ40p53, a murine cDNA fragment that encodes residues 41 to 387 was amplified by using the 5′ end primer Δ40p53 (AGAATTCACCATGGACGATCTGTTGCTGCC) and the 3′ end primer mC387. To generate p53AS, a murine cDNA fragment that encodes an alternative splice form of p53 was generated by using the 5′ end primer 5mp53 and the consecutive 3′ end primers AS-up (TTGATCAAGGCTTGGAAGGCTCTAGGCTGGAGGCTGGAGTGAGCCCTGCT) and AS-dn (TGAATTCCTAGCAGTTTGGGCTTTCCTCCTTGATCAAGGCTTGGAAG).

cDNAs encoding murine p63 isoforms, p63α, ΔNp63α, p63γ, and ΔNp63γ were kindly given by C. Di Como (20). All p63 proteins were N-terminally Myc-tagged as previously described (20). All p63-expressing constructs were confirmed by DNA sequencing. cDNAs encoding human p73 isoforms, p73α, ΔNp73α, p73β, and ΔNp73β were as previously described (35).

The luciferase reporter constructs under the control of the p21 promoter, IGFBP3 box A, and IGFBP3 box B were as previously described (5, 41). Luciferase reporter constructs under the control of the IGFBP3 promoter, pGL2/−1165 IGFBP3 and pGL2/−705 IGFBP3, were kindly given by Xiao-Fan Wang. Deletion constructs of the IGFBP3 promoter in the luciferase reporter vector pGL2 were generated by PCR and confirmed by sequencing. To generate pGL2/−256 IGFBP3, a genomic fragment of the IGFBP3 promoter spanning nucleotides (nt) −256 to +72, with +1 being the transcriptional start site, was amplified by using the 5′ end primer −256 BP3 (AGGTACCTGGCCGGGCACACCTTG) and the 3′ end primer GLprimer2 (Promega). pGL2/−183 IGFBP3, pGL2/−116 IGFBP3, and pGL2/−91 IGFBP3 were amplified by using the 5′ end primers −183 BP3 (AGGTACCCGGGCGAGTCTCGAGCTG), −116 BP3 (AGGTACCCAGCCGTGCCTGCGCCGA), or −91 BP3 (AGGTACCCCTCCCAACCCCCACTCC) with the 3′ end primer GLprimer2.

To generate constructs expressing small interfering RNAs (siRNAs) targeting the IGFBP3 coding region, pSuper-si-IGFBP3-CR1 and pSuper-si-IGFBP3-CR2 64-nt oligonucleotides were annealed and cloned into pSuper. With the siRNA targeting region shown in bold, the oligonucleotides for pSuper-si-IGFBP3-CR1 were the forward oligonucleotide 5′-**GATCCCCGAGCACAGATACCCAGAACTTCAAGAGAGTTCTGGGTATCTGTGCTC**TTTTTGGAAA-3′ and the reverse oligonucleotide 5′-AGCTTTTCCAAAAA**GAGCACAGATACCCAGAAC**TCTCTTGAA**GTTCTGGGTATCTGTGCTC**GGG-3′. The oligonucleotides for pSuper-si-IGFBP3-CR2 were the forward oligonucleotide 5′-GATCCCC**GGGGAAGGAGGACGTGCAC**TTCAAGAGA**GTGCACGTCCTCCTTCCCC**TTTTTGGAAA-3′ and the reverse oligonucleotide 5′-AGCTTTTCCAAAAA**GGGGAAGGAGGACGTGCAC**TCTCTTGAA**GTGCACGTCCTCCTTCCCC**GGG-3′. The oligonucleotides for the control siRNA targeting rat p53 were the forward oligonucleotide 5′-GATCCCC**GAGCATTGCCCGGAGCTGC**TTCAAGAGA**GCAGCTCCGGGCAATGCTCC**TTTTTGGAAA-3′ and the reverse oligonucleotide 5′-AGCTTTTCCAAAAA**GAGCATTGCCCGGAGCTGC**TCTCTTGAAG **CAGCTCCGGGCAATGCTCC**GGG-3′.

Cell lines. The culture, transfection, and generation of MCF7 cell lines were performed as previously described (70). Individual clones were screened for the inducible expression of HA-tagged p53(ΔBD) by Western blot analysis with monoclonal antibodies against p53. Individual clones were screened for the inducible expression of Myc-tagged p63γ and Myc-tagged ΔNp63γ by Western blot analysis with monoclonal antibodies against Myc. The MCF7 cell lines p53-24, p53(ΔAD1ΔBD)-15, p63α-11, ΔNp63α-9, p73α-2, and p73β-31 were as previously described (13, 68, 69). EB and H1299 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

Luciferase assay. A dual luciferase reporter assay was used to determine the transcriptional activity of p53, engineered p53 mutants, and p53 family isoforms. The luciferase reporter constructs used were pGL2/p21-A (41), which is regulated by the p21 promoter with two p53-responsive elements; pGL2/IGFBP3-Box A (5), which contains the p53-responsive element within intron 1 of the IGFBP3 gene; pGL2/IGFBP3-Box B (5), which contains the p53-responsive element within intron 2 of the IGFBP3 gene; and pGL2/−1165 IGFBP3, pGL2/−705 IGFBP3, pGL2/−256 IGFBP3, pGL2/−183 IGFBP3, pGL2/−116 IGFBP3, and pGL2/−91 IGFBP3, which are regulated by the IGFBP3 promoter. In all, 1 μg of a luciferase reporter, 1 μg of pcDNA3 control vector or pcDNA3 vector that expresses p53 family isoforms, and 25 ng of *Renilla* luciferase assay vector pRL-CMV (Promega, Madison, Wis.) were cotransfected by using the calcium phosphate method into MCF7, H1299, or EB cells. A dual luciferase assay was performed in triplicate according to the manufacturer's instructions (Promega). The fold increase in relative luciferase activity is a product of the luciferase activity induced by p53 family isoforms divided by that induced by an empty pcDNA3 vector.

Western blot analysis. Western blot analysis was performed as previously described (70) with anti-p53 monoclonal antibodies DO-1, PAb1801, PAb240, and PAb421; antiactin polyclonal antibody (Sigma); anti-p21 polyclonal antibody (C-19) (Santa Cruz); anti-HA polyclonal antibody (Y11) (Santa Cruz); anti-HA monoclonal antibody (HA.11) (Covance); and anti-Myc monoclonal antibody (9B11) (Cell Signaling). To analyze IGFBP3 protein, conditioned medium was collected from MCF7 cells induced or uninduced to express p53 and mutants for 72 h. The conditioned medium was concentrated by using a VivaSpin 6-ml concentrator (Vivascience), resuspended with 2× sodium dodecyl sulfate sample buffer, and boiled for 5 min. Western blot analysis was then performed as described by using anti-IGFBP3 polyclonal antibody (Upstate).

RNA isolation, Northern blot analysis, and reverse transcription-PCR (RT-PCR). Total RNA was isolated by using Trizol reagents (Invitrogen). Northern blot analyses were performed as described (70). The p21, glyceraldehyde-3-phosphate dehydrogenase (GADPH), and aquaporin 3 probes were prepared as previously described (66, 70); the IGFBP3 cDNA probe was made from a 700-bp EcoRI-XhoI fragment. First-strand cDNA was synthesized by using iScript (Bio-Rad) according to the manufacturer's instructions. The level of the transcripts for IGFBP3 and GADPH were determined by PCR. The primers used to amplify GAPDH were as previously described (9). The primers used to amplify a 942-bp IGFBP3 cDNA fragment were the 5′ end primer 5-BP3-CR (AGAATTCTGT ACTGTCGCCCCATCC) and 3′ end primer 3-BP3-CR (AGAATTCCGTCTA CTTGCTCTGCATGC).

ChIP assay. A chromatin immunoprecipitation (ChIP) assay was performed as previously described (36). After 24 h of induction (+) or no induction (−) of full-length p53 or p53(ΔAD1ΔBD) in MCF7 cells, chromatin was cross-linked in 1% formaldehyde in phosphate-buffered saline (PBS), and nuclei were extracted. Chromatin was sonicated to yield 500- to 1,000-bp DNA fragments and immunoprecipitated with a mixture of anti-HA polyclonal antibody (Y11) (Santa Cruz) and anti-p53 PAb1801 monoclonal antibody. After reverse cross-linking and phenol-chloroform extraction, DNA fragments bound by p53 were purified over a QIAGEN column. PCR was performed to visualize the enriched DNA fragments. Primers designed to amplify the 11 decamers of the p53-responsive element within the IGFBP3 promoter were the 5′ end primer −282BP3 (TGC TGAGGTGGCCTGGAGT) and the 3′ end primer +51BP3 (TCCAGGCAGG AAGCGGCTGATC). Primers that amplify the 5′ p53-responsive element within the p21 promoter were as previously described (36).

Trypan blue dye exclusion assay. MCF7-p53-24 or MCF7-p53(ΔAD1ΔBD)-15 cells were seeded at a density of 2×10^4 cells/well in 24-well plates in the presence or absence of tetracycline. At 12 h after plating, anti-rabbit polyclonal antibody (Sigma) or anti-IGFBP3 polyclonal antibody (Upstate) was added to the cell culture supernatant at a dilution of 1:250. At 24 h after antibody addition, both floating cells in the medium and live cells on the plate were collected and concentrated by centrifugation. After staining with trypan blue (Sigma) for 10 min, both live (unstained) and dead (stained) cells were counted in a hemocytometer. The percentage of dead cells was calculated as the number of dead cells divided by the total number of cells counted.

Proliferation assays. A CellTiter96 nonradioactive cell proliferation assay (Promega) was used to perform an MTT [3-(4,5-dimethylthiazol-2-yl)2 2,5-diphenyl tetrazolium bromide] assay. MCF7-p53(ΔAD1ΔBD)-15 cells were seeded at a density of 5×10^3 cells/well in 96-well plates in the presence and absence of tetracycline. To use IGFBP3 siRNA to characterize the contribution of IGFBP3 during MCF7-p53(ΔAD1ΔBD)-15-mediated cell death, after adherence to the plate, cells were transfected with 50 ng (per 96-well plate) of either a control siRNA expression vector targeting rat p53 or a mix of siRNA expression vectors targeting two locations within the coding region of IGFBP3 by using FuGENE 6 transfection reagent (Roche). At 44 h after plating, cells were labeled with the dye solution for 4 h and permeabilized for at least 1 h, and the absorbance at 570 nm was read by a microplate reader. To use IGFBP3 neutralizing antibody, at 18 h after plating, anti-rabbit polyclonal antibody (Sigma) or anti-IGFBP3 polyclonal antibody (Upstate) was added to the cell culture supernatant at a dilution of 1:250. At 72 h after plating, samples were processed as above, and absorbance at 570 nm was determined. *P* values were determined by a Student's *t* test.

A CellTiter-Glo luminescent cell viability assay (Promega) was used to determine the cellular ATP content. MCF7-p53(ΔAD1ΔBD)-15 cells were transfected with pSuper empty vector or a mix of siRNA expression vectors targeting two locations within the coding region of IGFBP3 by using FuGENE 6 transfection reagent (Roche). At 24 h after transfection, cells were seeded at a density of 5×10^3/well in 96-well plates in the presence and absence of tetracycline. At 36 h after plating, the CellTiter-Glo luminescent cell viability assay (Promega) was performed according to the manufacturer's instructions. The relative luciferase unit is expressed as a percentage of the product of luciferase activity induced by ATP content in the presence of p53 divided by that induced in the absence of p53. *P* values were determined by a Student's *t* test.

Drug treatments. To verify that cisplatin stabilizes endogenous p53 and that trichostatin A (TSA) increases acetylation of histones, MCF7 cells were treated with 50 μM cisplatin (Sigma) dissolved in H_2O and 100 ng of TSA (Upstate) per ml dissolved in 75% ethanol. To determine the effect upon IGFBP3 expression, MCF7 cells were treated with 50 μM cisplatin and 100 ng of TSA per ml for 12 h. To determine whether exogenous p53, p63α, or p73α induces IGFBP3 in the presence of TSA, MCF7 cells were induced or uninduced to express p53, p63α, or p73α for 12 h prior to treatment with 100 ng of TSA per ml for another 12 h.

Affymetrix gene chip analysis. Total RNA from MCF7 cells induced and uninduced to express full-length p53 and p53(ΔAD1ΔBD) was isolated, labeled, and hybridized to an Affymetrix gene chip (U133 plus 2.0).

RESULTS

IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53. p53 contains several functional domains (Fig. 1A). During the characterization of these domains by using stable inducible cell lines, we have previously reported that p53 (ΔAD1ΔBD), which lacks the N-terminal AD1 and the C-terminal BD, is more potent than full-length p53 to induce apoptosis in MCF7 breast adenocarcinoma cells (69). We chose MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cell lines because the proteins expressed by these lines are equivalent, although they are expressed at levels twofold higher than endogenous p53 levels induced by cisplatin (Fig. 1B and data not shown). While the BD has been characterized as a negative regulatory domain in vitro (23, 25, 56), to date, no one has been able to demonstrate the negative activity of this domain in vivo. We reasoned that the study of p53 (ΔAD1ΔBD) might shed insight into how the BD functions as a negative regulator. Interestingly, we found that IGFBP3, a proapoptotic target gene, was induced by p53(ΔAD1ΔBD) but not by full-length p53, as analyzed by Northern blotting (Fig. 1C). To demonstrate that both full-length p53 and p53 (ΔAD1ΔBD) are transcriptionally active, we analyzed the expression of p21. Both full-length p53 and p53(ΔAD1ΔBD) were able to induce p21. GAPDH was used as a loading con-

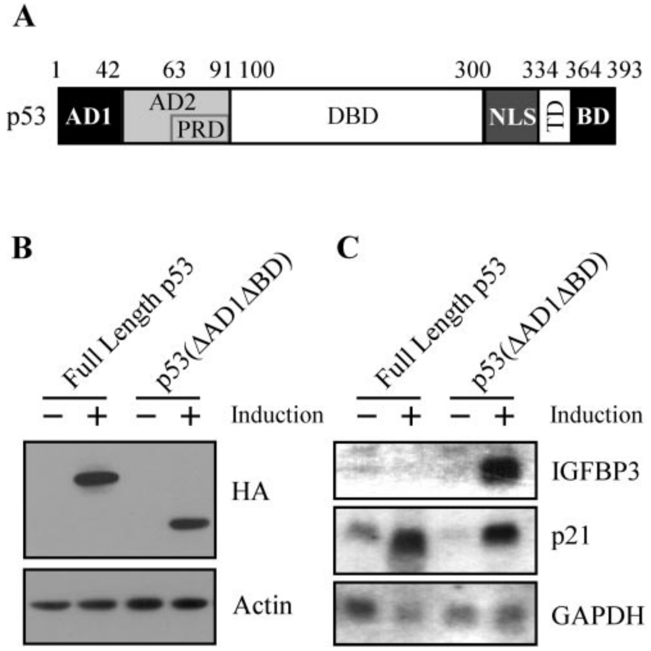


FIG. 1. IGFBP3 expression is induced by p53(ΔAD1ΔBD), but not by full-length p53. (A) Schematic representation of p53 functional domains: AD1, AD2, PRD, sequence-specific DBD, the nuclear localization signal (NLS), TD, and the C-terminal BD. Numbers indicate amino acid residues. (B) The level of p53 and actin was assayed by Western blot analysis by using anti-HA monoclonal and antiactin polyclonal antibodies, respectively, in MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cells in the absence (−) or presence (+) of p53 or p53(ΔAD1ΔBD) for 24 h. (C) A Northern blot was prepared by using total RNAs isolated from MCF7-HA-p53-24 and MCF7-HA-p53(ΔAD1ΔBD)-15 cells in the absence (−) or presence (+) of p53 or p53(ΔAD1ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes.

trol. These data suggest that AD1 and the BD inhibit the ability of full-length p53 to induce IGFBP3.

IGFBP3 was shown to be regulated by p53 in EB-1 cells, a colorectal carcinoma cell line that expresses exogenous p53 under control of the metallothionein promoter (5). Because we identified a unique situation where p53(ΔAD1ΔBD), but not full-length p53, induced IGFBP3, we wanted to characterize the mechanism regulating the ability of full-length p53 to induce IGFBP3 in MCF7 cells. Interestingly, O-glycosylation masks the PAb421 epitope of p53 in EB-1 cells and the resulting O-glycosylated p53 is activated for DNA binding in vitro (57).

The IGFBP3 promoter is activated by p53(ΔAD1ΔBD) but not by full-length p53. The canonical p53-responsive element contains two decamers [RRRC(A/T)(A/T)GYYY] separated by a spacer of 0 to 13 bp, where R represents purine and Y represents pyrimidine (15). IGFBP3 is a unique p53 target gene in that it contains 11 tandem decamers of the p53-responsive element within the proximal promoter, with each decamer separated by ≤9 bp (3). Thus, the IGFBP3 promoter potentially contains 10 tandem canonical p53-responsive elements spanning the region from nt −246 to −92, with +1 as the transcriptional start site. In addition, IGFBP3 contains two canonical p53-responsive elements, called box A and box B, within introns 1 and 2, respectively (5) (Fig. 2A).

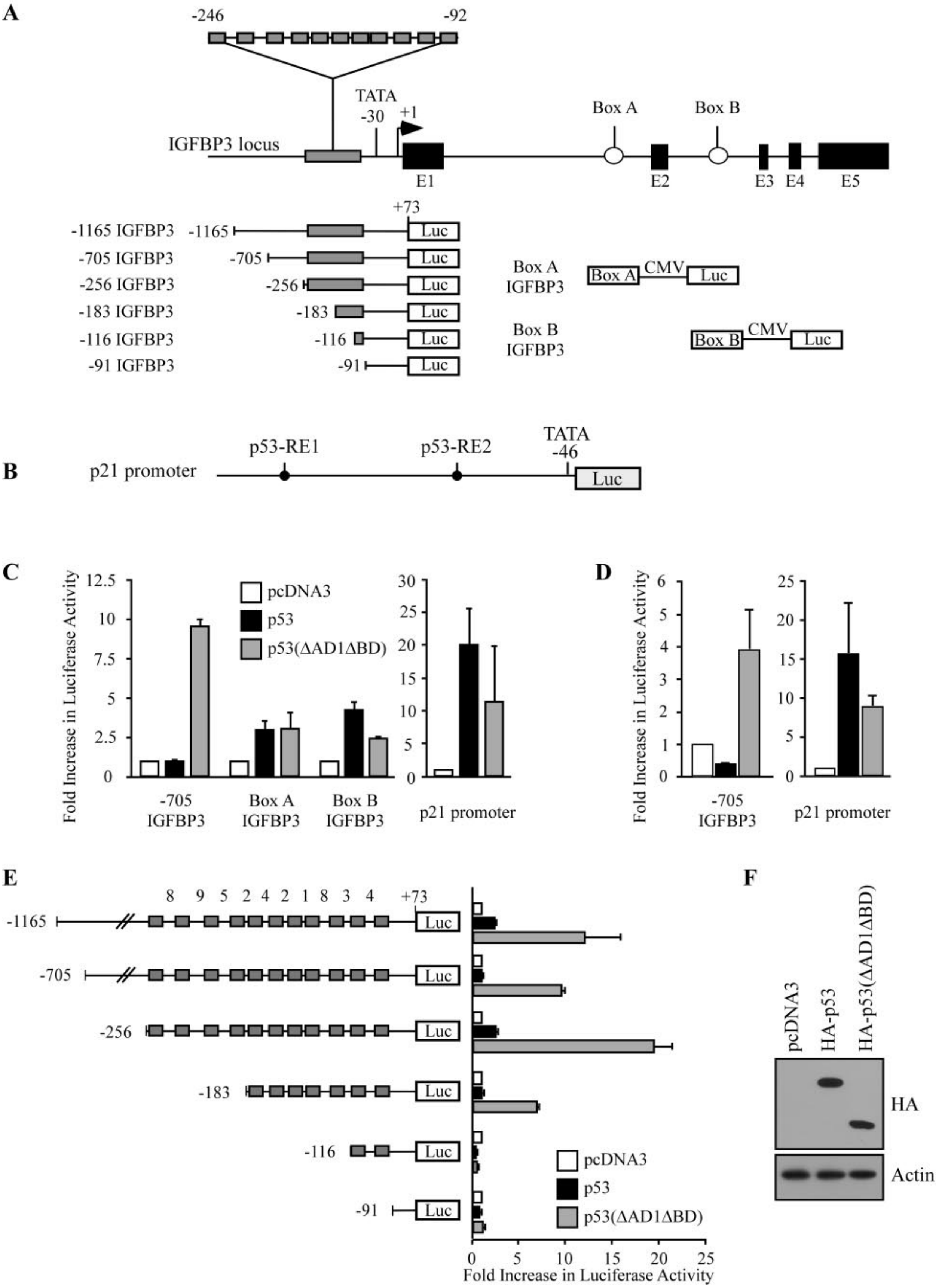
To determine which regions within the IGFBP3 gene are responsive to p53(ΔAD1ΔBD), we analyzed the ability of transiently overexpressed HA-p53(ΔAD1ΔBD) and full-length HA-p53 to activate the IGFBP3 promoter as well as box A and box B in MCF7 cells by using a dual luciferase reporter assay. We found that the −1165 and −705 IGFBP3 reporter constructs (Fig. 2A), which contain the IGFBP3 promoter from nt −1165 to +73 and from nt −705 to +73, respectively, were activated by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 2C and E). Surprisingly, reporters under the control of box A or box B linked to a minimal cytomegalovirus promoter (Fig. 2A) were only weakly activated by p53(ΔAD1ΔBD) and full-length p53 (Fig. 2C). The luciferase reporter under the control of the p21 promoter with two canonical p53-responsive elements (Fig. 2B) was activated by both full-length p53 and p53(ΔAD1ΔBD), albeit to a lesser extent by the latter (Fig. 2C). Equal levels of p53 protein are expressed by HA-p53 and HA-p53 (ΔAD1ΔBD) as detected by Western blot analysis (Fig. 2F). These data suggest that the p53-responsive elements within the IGFBP3 promoter are primarily responsible for the induction of IGFBP3 by p53(ΔAD1ΔBD).

To determine whether our observation was cell type specific, we tested the ability of full-length p53 and p53(ΔAD1ΔBD) to activate the IGFBP3 promoter in EB colon and H1299 non-small cell lung carcinoma cells. We found the same pattern of activation in EB and H1299 cells: the IGFBP3 promoter was activated by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 2D and data not shown). Again, the p21 promoter was activated by both p53(ΔAD1ΔBD) and full-length p53 (Fig. 2D and data not shown).

Because IGFPB3 is unique in that the promoter contains 11 tandem decamers of the p53-responsive element, we wanted to determine which decamers were necessary or sufficient for activation by p53(ΔAD1ΔBD). Thus, we generated four new reporter constructs: −256 IGFBP3, −183 IGFBP3, −116 IGFBP3, and −91 IGFBP3 (Fig. 2A). We found that the −256 IGFBP3 reporter, which contains all 11 decamers, was strongly activated by p53(ΔAD1ΔBD) (Fig. 2E). Surprisingly, we found that the 11 decamers function as a unit. Deletion of four upstream decamers inhibited activation of the −183 IGFBP3 reporter by p53(ΔAD1ΔBD) (Fig. 2E). In addition, the presence of only two decamers, recapitulating the canonical p53-responsive element, was not sufficient for activation of the −116 IGFBP3 reporter by p53(ΔAD1ΔBD) (Fig. 2E). Similarly, no activity was detected when all 11 decamers were deleted as in the −91 IGFBP3 reporter (Fig. 2E). Consistent with the above data, full-length p53 did not strongly activate any of the reporter constructs (Fig. 2E). Taken together, these data and results obtained by Northern blot analysis (Fig. 1C) indicate that p53(ΔAD1ΔBD) but not full-length p53 is capable of transactivating the IGFBP3 gene.

Activation of the IGFBP3 promoter by p53 requires the presence of AD2 and deletion of the BD. The mechanism by which p53 induces a specific activity over another remains unclear in p53 research. We reasoned that p53 functional domains may play a role in target gene selection. To this end, we used deletion and point mutation of p53 to analyze the requirements of p53 functional domains for activation of the IGFBP3 promoter.

Because p53(ΔAD1ΔBD) lacks two functional domains, we


A
-246
-92
TATA
-30
+1
Box A
Box B
IGFBP3 locus
E1
E2
E3
E4
E5
+73
-1165 IGFBP3
-1165
Luc
-705 IGFBP3
-705
-256 IGFBP3
-256
-183 IGFBP3
-183
-116 IGFBP3
-116
-91 IGFBP3
-91
Box A IGFBP3
Box A
CMV
Box B IGFBP3
Box B
B
p21 promoter
p53-RE1
p53-RE2
TATA
-46
C
Fold Increase in Luciferase Activity
12.5
10
7.5
5
2.5
0
pcDNA3
p53
p53(ΔAD1ΔBD)
-705 IGFBP3
Box A IGFBP3
Box B IGFBP3
30
25
20
15
p21 promoter
D
6
4
3
2
1
E
8 9 5 2 4 2 1 8 3 4
F
pcDNA3
HA-p53
HA-p53(ΔAD1ΔBD)
HA
Actin
Fold Increase in Luciferase Activity

first wanted to determine which domain is inhibitory. We found that deletion of only AD1 resulted in activity similar to full-length p53 (Fig. 3A); however, deletion of only the BD enabled strong activation of the IGFBP3 promoter (Fig. 3A). We then wanted to identify the inhibitory residues within the BD. We found that p53(ΔC20), which lacks the C-terminal 20 amino acids, strongly activated the IGFBP3 promoter, albeit less than p53(ΔBD) (Fig. 3A). p53(ΔC10), which lacks the C-terminal 10 amino acids, had activity similar to that of full-length p53 (Fig. 3A). p53(R249S), a tumor-derived DNA binding mutant, served as a negative control and did not activate the IGFBP3 promoter. p53 protein was expressed by all p53 constructs used in these studies (data not shown).

Next, we wanted to characterize the N-terminal requirement. We reasoned that AD1 is not required because p53 (ΔAD1ΔBD) strongly activates the IGFBP3 promoter. To test this, we used a double point mutation in AD1 (Gln^{22}-Ser^{23}), resulting in a well-defined AD1-deficient mutant ($\mathrm{AD1}^{-}$) that is incapable of interacting with the transcriptional machinery (34). We found that p53($\mathrm{AD1}^{-}$ΔBD), which contains the nonfunctional AD1 and lacks the BD, was not able to activate the IGFBP3 promoter, whereas p53(ΔBD), which lacks only the BD, was active (Fig. 3B). Thus, AD1 is dispensable, but an inactive AD1 is inhibitory. This finding suggests that the conformation of p53 might be important for the induction of IGFBP3.

Because AD1 is dispensable, we wanted to characterize the role of AD2. We have previously found that AD2 is important for the induction of apoptosis (70). Interestingly, we found that deletion of AD2 (ΔAD2) or mutation of AD2 ($\mathrm{AD2}^{-}$) abolished the activity of p53(ΔBD) at the IGFBP3 promoter (Fig. 3C). Further strengthening the importance of AD2 was the finding that mutation of AD2 ($\mathrm{AD2}^{-}$) abolished the activity of p53(ΔAD1ΔBD) at the IGFBP3 promoter (Fig. 3C). Taken together, these data suggest that a functional AD2 is required for activation of the IGFBP3 promoter.

The activity of naturally occurring p53 isoforms correlates with the activity of our engineered p53 mutants. p53 has been reported to exist as both individual and combined N- and C-terminally truncated isoforms (Fig. 4A). To determine whether the behavior of our engineered p53 mutants represents the behavior of naturally occurring p53 isoforms, we generated untagged constructs expressing human and mouse p53 isoforms that have been reported to exist in the literature, namely human ΔNp53, mouse Δ40p53, human I9+p53, human p53 (ΔNΔBD), and mouse p53AS (Fig. 4A). Similar to p53(ΔAD1) which lacks the first 42 amino acids, human ΔNp53 lacks the first 39 amino acids. ΔNp53 is generated through alternative splicing of intron 2. The resulting exon contains three stop codons; thus, the translational start site of ΔNp53 is at codon 40 of full-length p53 (21). Mouse Δ40p53 lacks the first 40 amino acids and is generated through the use of an internal translational start site at codon 41 (reviewed in reference 11). As expected, the activity of ΔNp53, Δ40p53, and p53(ΔAD1) was analogous, with all constructs having similar levels of activity at the IGFBP3 promoter compared to full-length human and mouse p53 (Fig. 4B and C). I9+p53 is generated through alternative splicing of intron 9 and results in the substitution of 62 C-terminal amino acids by 10 unique amino acids (18). Because alternative splicing abolishes the TD, I9+p53 should be transcriptionally inactive. As expected, I9+p53 did not activate the IGFBP3 promoter (Fig. 4B). C-terminal cleavage of p53, detectable by the lack of anti-p53 PAb421 epitope located within amino acids 371 to 380, occurs through interaction with single-stranded DNA (ssDNA) or cleavage by calpain (42, 45). We reasoned that p53(ΔBD), which lacks amino acids 364 to 393, mimics a C-terminally cleaved p53. It is also likely that a p53 protein exists that is alternatively spliced at the N terminus and is subsequently truncated at the C terminus through interaction with ssDNA or calpain. Thus, we generated p53 (ΔNΔBD). As expected, the activities of p53(ΔNΔBD) and p53 (ΔAD1ΔBD) were analogous, with both constructs strongly activating the IGFBP3 promoter (Fig. 4B). In addition, we found that mouse p53AS activated the IGFBP3 promoter, whereas full-length mouse p53 did not (Fig. 4C). p53AS is generated through alternative splicing of intron 10 and results in the substitution of 26 C-terminal amino acids by 17 unique amino acids. Mouse p53(A135V), a naturally occurring DNA binding mutant, was inactive and served as a negative control (Fig. 4C). p53 protein was expressed by these constructs as detected by Western blot analysis (Fig. 4D and E). Taken together, these data suggest that naturally occurring p53 isoforms differentially regulate IGFBP3. Unfortunately, we were not able to detect endogenous levels of naturally occurring isoforms by Western blot analysis. However, we cannot rule out the possibility that undetectable levels exist and may possess significant function.

IGFBP3 expression is induced by C-terminally truncated p63 and p73 isoforms, p63γ and p73β, but not by full-length ones. Since the C terminus of p53 plays a critical role in regulating IGFBP3, we reasoned that the C termini of p63 and p73 might also affect the regulation of IGFBP3. Both p63 and p73 undergo alternative splicing of their C termini, resulting in three p63 isoforms (α to γ) and seven p73 isoforms (α to η) (Fig. 5A). These isoforms, called the TA and ΔN isoforms, are transcribed from the upstream promoter as well as from a cryptic promoter within intron 3, respectively. Thus, many nat-

FIG. 2. Activation of the IGFBP3 promoter by p53(ΔAD1ΔBD) requires all 11 decamers of the p53-responsive element. (A) Schematic representation of the IGFBP3 locus and luciferase reporter constructs. CMV, cytomegalovirus. (B) Schematic representation of the p21 luciferase reporter construct. (C) The IGFBP3 promoter is activated by p53(ΔAD1ΔBD) but not by full-length p53 in MCF7 cells. MCF7 cells were cotransfected with 1 μg of the luciferase reporter under control of the IGFBP3 promoter, box A, or box B and 1 μg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). (D) The IGFBP3 promoter is activated by p53(ΔAD1ΔBD) but not by full-length p53 in EB cells. The experiment was performed as described above except that EB cells were used. (E) The 11 decamers of the p53-responsive element function as a unit. MCF7 cells were cotransfected with 1 μg of a luciferase reporter under control of various lengths of the IGFBP3 promoter and 1 μg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). The number between each decamer indicates the number of base pairs of separation. (F) HA-p53 and HA-p53(ΔAD1ΔBD) are expressed at equal levels. MCF7 cells were transfected with 5 μg of empty pcDNA3 or pcDNA3 vector expressing HA-p53 or HA-p53(ΔAD1ΔBD). HA-p53 and HA-p53(ΔAD1ΔBD) were detected with anti-HA monoclonal antibody. Actin was detected with antiactin polyclonal antibody.

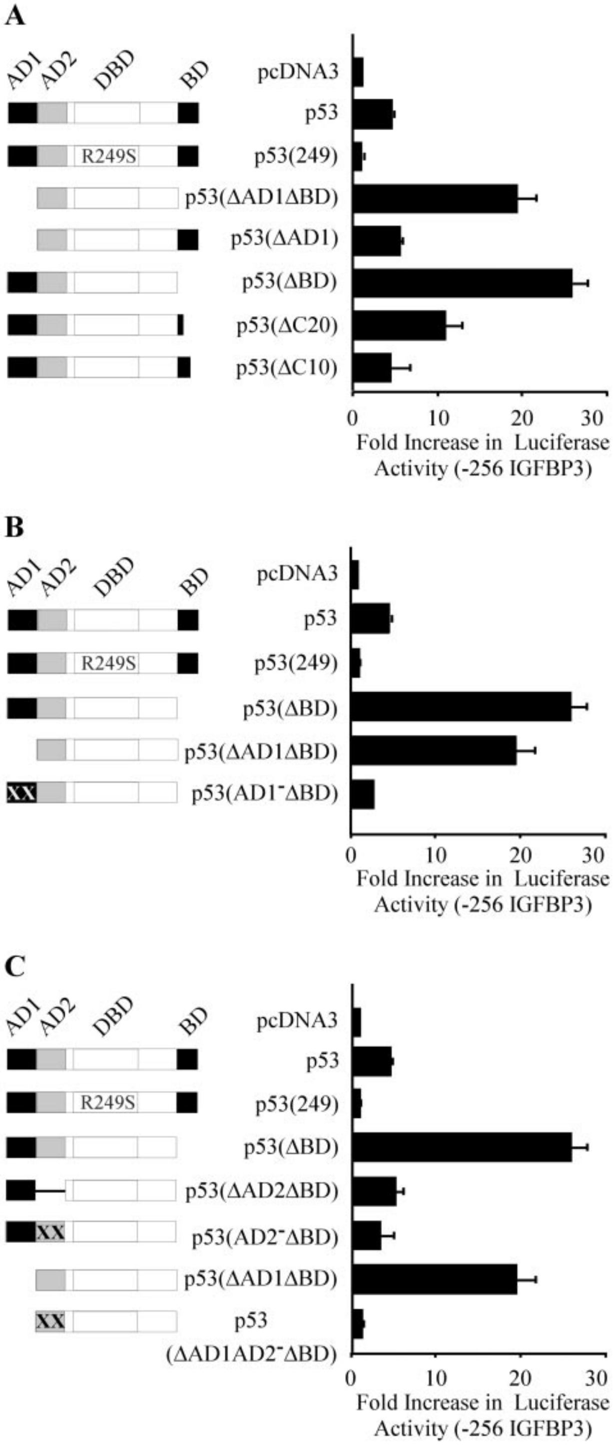


FIG. 3. Activation of the IGFBP3 promoter by p53 requires the presence of AD2 and deletion of the BD. Schematic representations of p53 and mutants are shown at left. Graphs (right) show increases (*n*-fold) in activation of the −256 IGFBP3 reporter by p53 and mutants. MCF7 cells were cotransfected with 1 μg of luciferase reporter under control of the IGFBP3 promoter and 1 μg of empty pcDNA3 or pcDNA3 vector expressing p53 and various mutants. (A) The BD inhibits p53 activation of the IGFBP3 promoter. (B) AD1 is dispensable, but an inactive AD1 is inhibitory for p53 activation of the IGFBP3 promoter. (C) AD2 is required for activation of the IGFBP3 promoter.

urally occurring p63 and p73 proteins are generated through alternative splicing and the use of two transcriptional start sites.

To determine whether the N and/or the C termini of p63 and p73 modulate the regulation of IGFBP3, previously characterized stable MCF7 cell lines were analyzed for their ability to regulate IGFBP3 as follows: for cell lines inducibly expressing the Myc-tagged p63 isoforms, p63α (clone 11), ΔNp63α (clone 9), p63γ (clone 19), or ΔNp63γ (clone 18) was used; for cell lines expressing the HA-tagged p73 isoforms, p73α (clone 2) or p73β (clone 31) was used. Equal levels of p63 are expressed by the representative clones as detected by Western blot analysis (Fig. 5B). Interestingly, we found that IGFBP3 was strongly induced by the C-terminally truncated isoforms p63γ and p73β but not by the full-length or the ΔN isoforms, as detected by Northern blotting (Fig. 5C). We also analyzed the ability of the p63 and p73 isoforms to regulate the well-characterized p53 target gene, p21. Similar to the regulation of IGFBP3 by p63 and p73 isoforms, the C-terminally truncated isoforms p63γ and p73β strongly induce p21. In addition, p21 is induced by full-length p73α, albeit weakly. Thus, a pattern emerges for p53 family regulation of IGFBP3: the C terminus of the p53 family is inhibitory for the induction of IGFBP3.

p63γ and p73β activate the IGFBP3 promoter. To determine which regions within the IGFBP3 gene are responsive to p63γ and p73β, we analyzed the ability of various p63 and p73 isoforms to activate the IGFBP3 promoter as well as box A and box B in MCF7 cells by using a dual luciferase reporter assay. We found that the −705 IGFBP3 reporter was activated by p63γ but not by p63α, ΔNp63α, or ΔNp63γ (Fig. 5D). None of the p63 isoforms analyzed strongly activated box A or box B (Fig. 5D). Similarly, the −705 IGFBP3 reporter was activated by p73β but not by p73α, ΔNp73α, or ΔNp73β. None of the p73 isoforms analyzed strongly activated box A or box B (Fig. 5E). Next, we wanted to determine which decamers within the IGFBP3 promoter were necessary or sufficient for activation by p63γ and p73β. We found that all 11 decamers were required for activation of the IGFBP3 promoter. Deletion of four upstream decamers, as with the −183 IGFBP3 construct, severely inhibited p63γ and p73β activity at the IGFBP3 promoter (Fig. 5F). No activity was detected when 9 or all 11 decamers were deleted, as with the −116 IGFBP3 or −91 IGFBP3 construct, respectively (Fig. 5F). Thus, we found that both p63γ and p73β behave similarly to p53(ΔAD1ΔBD). Protein was expressed by these p63 and p73 constructs as detected by Western blotting (Fig. 5G and 5H). Taken together, these data and results obtained by Northern blot analysis (Fig. 5C) indicate that p63γ and p73β, but not the α or ΔN isoforms, utilize the IGFBP3 promoter to induce IGFBP3.

IGFBP3 is an important effector of p53(ΔAD1ΔBD)-dependent cell death. Because p53(ΔAD1ΔBD) is more potent than full-length p53 to induce apoptosis in MCF7 cells (69) and IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 1C), we wanted to characterize the contribution of IGFBP3 in p53- and p53(ΔAD1ΔBD)-dependent apoptosis. To do this, we performed a trypan blue dye exclusion assay and found that cell death induced by p53(ΔAD1ΔBD) was reduced by 36% with the addition of the IGFBP3 neutralizing antibody to the cell culture supernatant compared to the cells incubated with the rabbit control antibody (Fig. 6A). Cell death induced by full-length p53 was not reduced by incubation with the

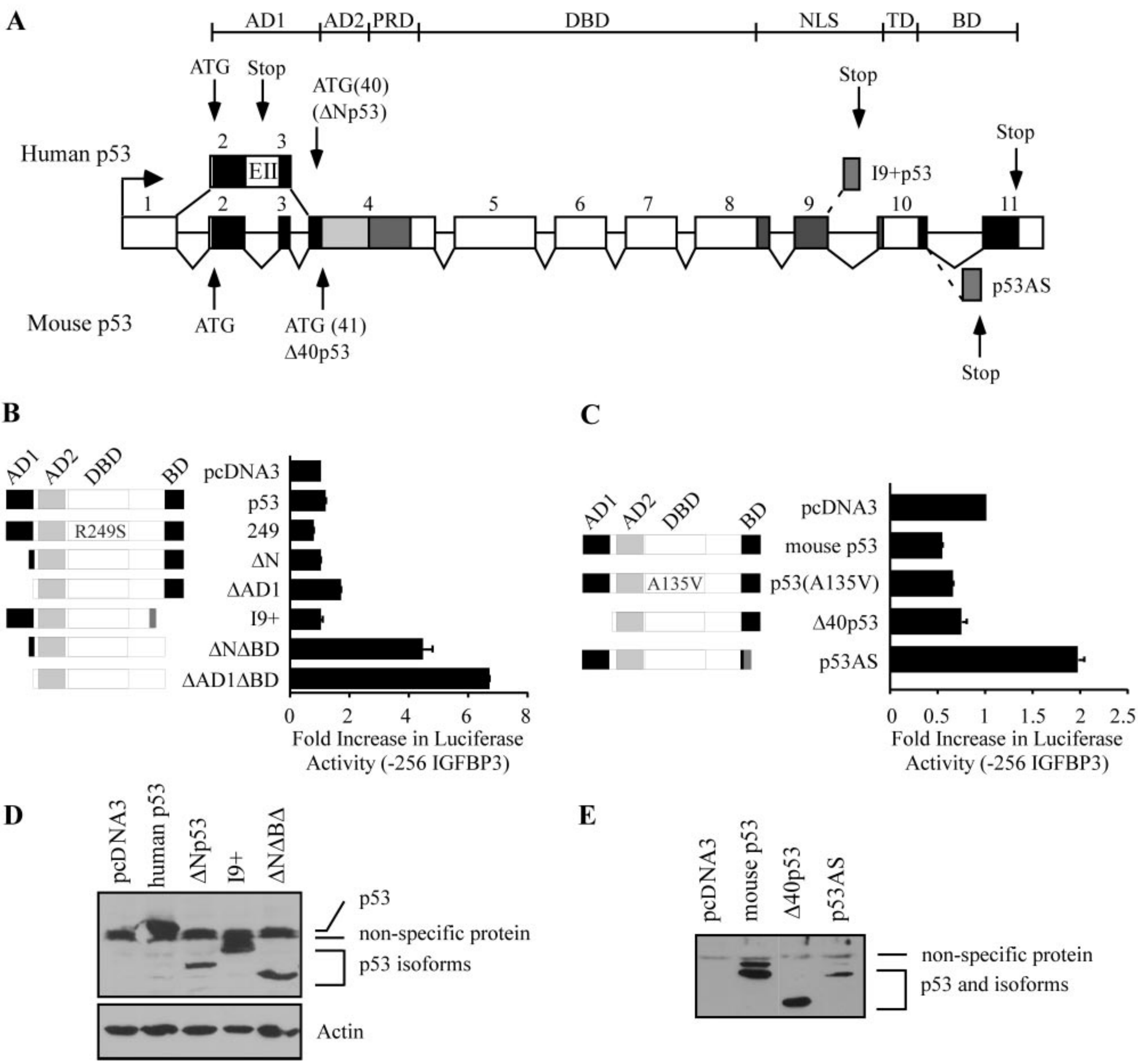
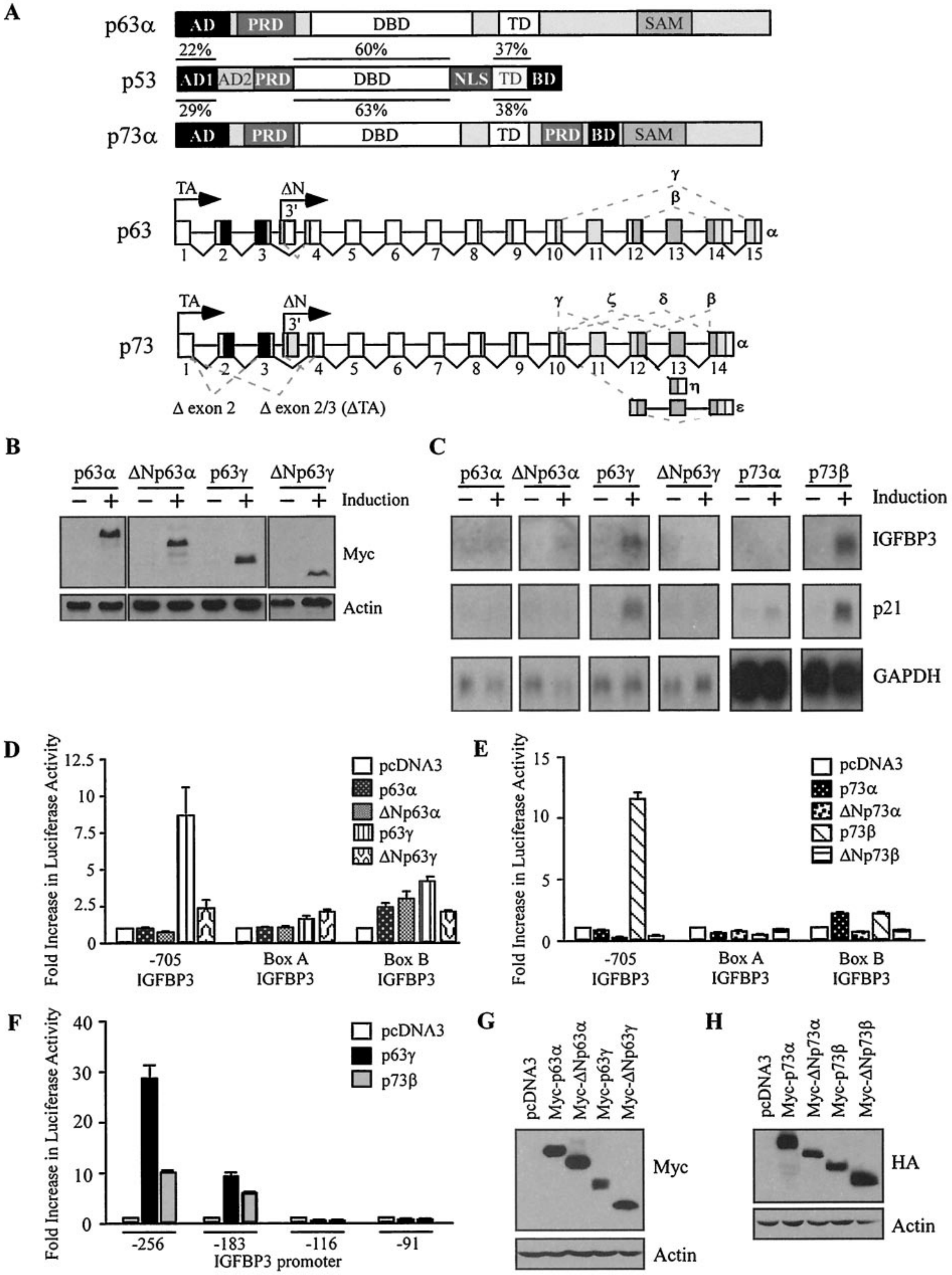


FIG. 4. The activity of naturally occurring p53 isoforms correlates with the activity of engineered mutants. (A) Schematic representation of human (top) and murine (bottom) p53 isoforms. Numbers indicate exons. Dotted lines indicate alternative splicing. Human ΔNp53 is generated through alternative splicing of intron 2. The alternative exon (EII) contains three stop codons; thus, translation begins at codon 40. Additionally, human ΔNp53 is generated through the use of an internal translational start site at codon 40. Mouse Δ40p53 is generated through the use of an internal translational start site at codon 41. Human I9+p53 and mouse p53AS are generated through alternative splicing of introns 9 and 10, respectively. (B) Naturally occurring human p53 isoforms activate the IGFBP3 promoter. Shown is a schematic representation of human p53 isoforms (left). MCF7 cells were cotransfected with 1 μg of luciferase reporter under control of the IGFBP3 promoter and 1 μg of empty pcDNA3 or pcDNA3 vector expressing p53 and various mutants (right). (C) Naturally occurring mouse p53 isoforms activate the IGFBP3 promoter. The schematic representation and graphs are analogous to those described for panel B. (D and E) p53 isoforms are expressed from various p53 constructs. MCF7 cells were transfected with empty pcDNA3 or pcDNA3 vector expressing various human p53 isoforms. H1299 cells were transfected with empty pcDNA3 or pcDNA3 vector expressing various mouse p53 isoforms. p53 was detected with anti-p53 monoclonal antibodies. Actin was detected with antiactin polyclonal antibody.

IGFBP3 antibody (Fig. 6A). In addition, we performed an MTT assay and found that incubation with the IGFBP3 neutralizing antibody increases the survival of cells expressing p53 (ΔAD1ΔBD) in comparison to cells incubated with the rabbit control antibody (Fig. 6B). To confirm these data, we measured cell viability in the presence of p53(ΔAD1ΔBD) with and without knockdown of IGFBP3 by siRNA. We used transient transfection of vector-expressed siRNA hairpins that target two locations within the IGFBP3 coding region. Using the MTT assay, we found that transient knockdown of IGFBP3 with siRNA increases the viability of cells expressing p53 (ΔAD1ΔBD) in comparison to cells transfected with control siRNA targeting rat p53 (Fig. 6C). In addition, we measured cellular ATP as an indication of cell viability and found that


A
p63α
AD
PRD
DBD
TD
SAM
22%
60%
37%
p53
AD1
AD2
PRD
DBD
NLS
TD
BD
29%
63%
38%
p73α
AD
PRD
DBD
TD
PRD
BD
SAM
p63
TA
ΔN
3'
γ
β
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
α
p73
TA
ΔN
3'
γ ζ δ β
1 2 3 4 5 6 7 8 9 10 11 12 13 14
α
η
ε
Δ exon 2
Δ exon 2/3 (ΔTA)
B
p63α ΔNp63α p63γ ΔNp63γ
− + − + − + − +
Induction
Myc
Actin
C
p63α ΔNp63α p63γ ΔNp63γ p73α p73β
− + − + − + − + − + − +
Induction
IGFBP3
p21
GAPDH
D
Fold Increase in Luciferase Activity
12.5
10
7.5
5
2.5
0
pcDNA3
p63α
ΔNp63α
p63γ
ΔNp63γ
-705 IGFBP3
Box A IGFBP3
Box B IGFBP3
E
Fold Increase in Luciferase Activity
15
10
5
0
pcDNA3
p73α
ΔNp73α
p73β
ΔNp73β
-705 IGFBP3
Box A IGFBP3
Box B IGFBP3
F
Fold Increase in Luciferase Activity
40
30
20
10
0
pcDNA3
p63γ
p73β
-256
-183
-116
-91
IGFBP3 promoter
G
pcDNA3
Myc-p63α
Myc-ΔNp63α
Myc-p63γ
Myc-ΔNp63γ
Myc
Actin
H
pcDNA3
Myc-p73α
Myc-ΔNp73α
Myc-p73β
Myc-ΔNp73β
HA
Actin

siRNA targeting IGFBP3 increases the viability of cells expressing p53(ΔAD1ΔBD) compared to cells transfected with empty pSuper vector (Fig. 6D). To demonstrate the extent of the transient knockdown of induced IGFBP3 levels, RT-PCR was performed on p53(ΔAD1ΔBD)-15 cells transiently transfected with a mix of the IGFBP3-siRNA expression vectors pSuper-si-IGFBP3-CR-1 and pSuper-si-IGFBP3-CR-2 or a control siRNA expression vector targeting rat p53 and were subsequently induced or uninduced to express p53(ΔAD1ΔBD). A 39% reduction of induced IGFBP3 levels was detected by RT-PCR (Fig. 6E). Although we cannot completely abolish induced IGFBP3 levels by using transient transfection of pSuper-si-IGFBP3-CR-1 and pSuper-si-IGFBP3-CR-2, we do detect a modest but reproducible reduction of cell death induced by p53(ΔAD1ΔBD) in the presence of IGFBP3 siRNA (Fig. 6C and D). We suspect that the reduction of cell death would be greater given more efficient IGFBP3 knockdown. Taken together, these data suggest that IGFBP3 is an important effector of p53-dependent apoptosis.

Full-length p53 and p53(ΔAD1ΔBD) bind to the IGFBP3 promoter in vivo. To gain insight into how the BD regulates p53 target gene selection, we wanted to characterize the mechanism that enables p53(ΔAD1ΔBD), but not full-length p53, to induce IGFBP3. First, we wanted to characterize the induction of IGFBP3 by p53(ΔBD), because deletion of the BD renders p53 capable of activating the IGFBP3 promoter (Fig. 3A). Thus, we generated several stable MCF7 cell lines inducibly expressing HA-tagged p53(ΔBD) in the tetracycline-repressible system. A representative cell line, clone 3, was selected for presentation. Western blot analysis showed that the level of p53(ΔBD) protein expressed in this line was comparable to that of full-length p53 and p53(ΔAD1ΔBD) in M7-p53-24 and M7-p53(ΔAD1ΔBD)-15 cell lines, respectively (Fig. 7A). To demonstrate that p53(ΔBD) is transcriptionally active, we analyzed the expression of p21. Like full-length p53 and p53(ΔAD1ΔBD), p53(ΔBD) was able to induce p21 (Fig. 7B). Surprisingly, we found that p53(ΔBD) did not significantly induce IGFBP3, as determined by Northern blot analysis (Fig. 7B). Although unexpected, our result is not unprecedented as deletion of the BD has been shown to activate DNA binding in vitro (25); however, to date deletion of the BD has not been shown to enhance the expression of a target gene. Western blot analysis further confirmed that p53(ΔBD) was not capable of inducing IGFBP3, since secreted IGFBP3 was substantially increased by p53(ΔAD1ΔBD) but not by full-length p53 or p53(ΔBD) (Fig. 7A). Although we do detect a slight increase in the secreted IGFBP3 protein level when full-length p53 or p53(ΔBD) is expressed, a corresponding increase in IGFBP3 mRNA level is not detected. Thus, we attribute the modest induction of IGFBP3 protein by full-length p53 and p53(ΔBD) to posttranslational stabilization of the IGFPB3 protein that may be effected by another p53 target gene. As hinted by the study of AD1, where AD1 is dispensable but an inactive AD1 is inhibitory (Fig. 3B), these data indicate that the conformation of the p53 protein is important for the regulation of IGFBP3.

To determine whether p53 DNA binding is the mechanism governing the regulation of IGFBP3 by p53, we performed ChIP assays. After a 24-h induction of full-length p53 or p53(ΔAD1ΔBD), chromatin was cross-linked, sonicated, and immunoprecipitated with a mixture of anti-HA polyclonal and anti-p53 PAb1801 monoclonal antibodies. To visualize the enriched DNA fragments, PCR was performed to amplify the region of the IGFBP3 promoter spanning the 11 decamers as well as the upstream p53-responsive element within the p21 promoter (Fig. 7C and D). Chromatin prepared without induction of full-length p53 or p53(ΔAD1ΔBD) was used as a negative control. We found that both full-length p53 and p53(ΔAD1ΔBD) bound to the IGFBP3 promoter (Fig. 7E); however, only p53(ΔAD1ΔBD) induces IGFBP3 (Fig. 1C). No enrichment of the DNA fragment containing the IGFBP3 promoter was detected in the samples not induced to express full-length p53 or p53(ΔAD1ΔBD). As expected, both full-length p53 and p53(ΔAD1ΔBD) bound to the p21 promoter (Fig. 7F). Thus, p53 DNA binding is not the mechanism by which IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53.

HDAC activity inhibits the ability of full-length p53 family isoforms to induce IGFBP3. Because full-length p53 binds the IGFBP3 promoter in vivo, we reasoned that under certain circumstances, full-length p53 would become competent to induce IGFBP3. Such circumstances might include the addition of an activating posttranslational modification, removal of an inhibitory posttranslational modification, dissociation from an inhibitory p53-associated protein, or a combination of these effects. To this end, we used MCF7 cells to characterize the regulation of IGFBP3 by endogenous p53 activated by the DNA damaging agent cisplatin in the presence and absence of the HDAC inhibitor TSA.

Western blotting showed that endogenous p53 protein was stabilized within 4 h upon DNA damage with 50 μM cisplatin, and the stabilization continued throughout 24 h (Fig. 8A). p21 protein was detected as early as 8 h after cisplatin treatment

FIG. 5. The C termini of p63 and p73 facilitate differential regulation of IGFBP3. (A) Schematic representation of p53 family functional domains and isoforms. SAM, sterile-α-motif domain; TA, isoforms transcribed from the upstream promoter; and ΔN, isoforms transcribed from the cryptic promoter within intron 3. Dotted lines indicate alternative splicing. Percent identity is indicated. (B) The levels of p63 and actin were assayed by Western blot analysis by using anti-Myc monoclonal and antiactin polyclonal antibodies, respectively, in MCF7-Myc-p63α-11, MCF7-Myc-ΔNp63α-9, MCF7-Myc-p63γ-19, and MCF7-Myc-ΔNp63γ-18 cells in the absence (−) or presence (+) of p63 for 24 h. (C) IGFBP3 is induced by p63γ and p73β but not by p63α, ΔNp63α, ΔNp63γ, or p73α. A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express various p63 or p73 isoforms for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes. (D and E) p63γ and p73β activate the IGFBP3 promoter. MCF7 cells were cotransfected with 1 μg of luciferase reporter under control of the IGFBP3 promoter, box A, or box B and 1 μg of empty pcDNA3 or pcDNA3 vector expressing various p63 (D) or p73 (E) isoforms. (F) The 11 decamers function as a unit in response to p63γ and p73β. (G and H) Proteins are expressed from various p63 and p73 constructs. MCF7 cells were transfected with 5 μg of empty pcDNA3 or pcDNA3 vector expressing Myc-tagged p63 isoforms or HA-tagged p73 isoforms. Levels of p63, p73, and actin were determined by Western blotting with anti-Myc monoclonal antibody, anti-HA monoclonal antibody, and antiactin polyclonal antibody, respectively.

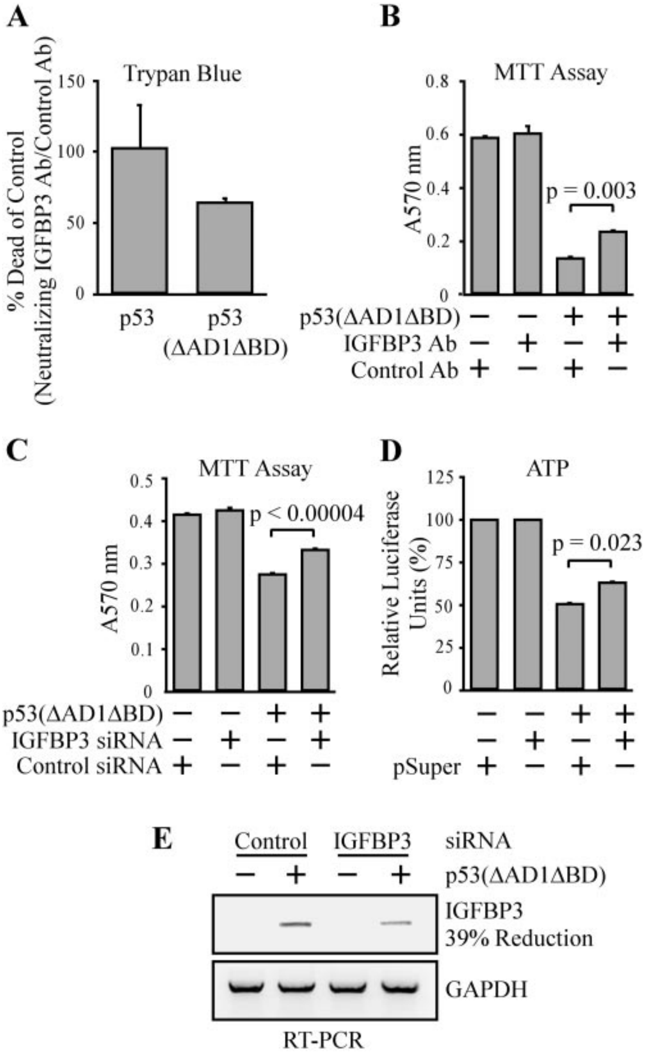


FIG. 6. IGFBP3 is an effector of p53(ΔAD1ΔBD)-mediated apoptosis. (A and B) Neutralizing IGFBP3 antibody reduces cell death induced by p53(ΔAD1ΔBD) but not by full-length p53. Results are shown of a trypan blue dye exclusion assay of MCF7-p53-24 and MCF7-p53 (ΔAD1ΔBD)-15 cells and MTT assay of MCF7-p53(ΔAD1ΔBD)-15 cells induced or uninduced to express the p53 protein in the presence of anti-IGFBP3 neutralizing antibody or anti-rabbit control antibody. Experiments were performed as described in Materials and Methods. (C and D) Knockdown of IGFBP3 by siRNA enhances viability of cells induced to express MCF7-p53(ΔAD1ΔBD)-15. Shown are the results of an MTT assay and an ATP luminescence assay of MCF7-p53(ΔAD1ΔBD)-15 cells induced or uninduced to express the p53 protein transiently transfected with vector-expressed siRNA against IGFBP3 (mix of two siRNAs targeting the coding region of IGFBP3) or with control siRNA against rat p53 or empty pSuper vector. Experiments were performed as described in Materials and Methods. (E) A 39% reduction of the p53(ΔAD1ΔBD)-induced IGFBP3 mRNA level by IGFBP3 siRNA. Levels of the transcripts for IGFBP3 and GAPDH with transient transfection of vectors expressing siRNA against IGFBP3 (mix of two siRNAs targeting the coding region of IGFBP3) or control siRNA against rat p53 were determined by RT-PCR with 28 cycles.

and increased throughout 24 h (Fig. 8A). HDAC inhibition, evidenced by increased levels of acetylated histone H3, was detected within 2 h upon treatment with 100 ng of TSA per ml, and the inhibition continued throughout 24 h (Fig. 8B). Because MCF7 cells did not tolerate a combined treatment of cisplatin and TSA for more than 12 h, we chose this time point for future analyses. We found that activation of p53 by cisplatin or inhibition of HDAC activity by TSA was not sufficient to induce IGFBP3, as detected by Northern blotting (Fig. 8C). However, the combined treatment with cisplatin and TSA enabled endogenous full-length p53 to adopt the conformation necessary to induce IGFBP3 (Fig. 8C). In contrast, p21 was induced upon DNA damage, but its expression was inhibited by the combined treatment (Fig. 8B and C).

To verify our observation that inhibition of HDAC activity restores the ability of endogenous p53 to induce IGFBP3, we tested the ability of exogenous full-length p53 and p73α to induce IGFBP3 in the presence of TSA. As noted previously, IGFBP3 was not induced by full-length p53 or p73α (Fig. 1C, 5C, and 8D). However, TSA treatment restored the ability of exogenous full-length p53 and p73α to induce IGFBP3 in the M7-p53-24 and M7-p73α-2 cell lines, respectively (Fig. 8D). Similarly, TSA treatment rendered full-length p63α competent to induce IGFBP3 (data not shown). We note that a slight increase in IGFBP3 mRNA was detected in the presence of TSA alone in M7-p53-24 and M7-p73α-2 cell lines (Fig. 8D). It is possible that a small amount of p53 or p73 was expressed in the uninduced condition, leading to the modest induction of IGFBP3 in the presence of TSA. Taken together, these data demonstrate that HDACs inhibit the ability of full-length p53 family proteins to induce IGFBP3 and that this inhibition can be overcome by combination treatments that simultaneously activate p53 and inhibit HDACs.

Differential target gene regulation by full-length p53 and p53(ΔAD1ΔBD). p53 family isoforms differentially regulate IGFBP3. Specifically, AD1 and the C-terminal BD inhibit induction of IGFBP3 by p53. Importantly, this inhibition can be overcome with inhibition of HDACs. To better understand the significance of this regulation, we wanted to determine whether other target genes are also under similar control by p53 isoforms. Thus, we performed Affymetrix gene chip analysis by using MCF7 cells induced and uninduced to express full-length p53 or p53(ΔAD1ΔBD). We identified several common target genes such as p21, FDXR, and others that were induced by full-length p53 and p53(ΔAD1ΔBD) (Table 1). We also identified several differentially regulated target genes that were induced by p53(ΔAD1ΔBD) but not by full-length p53 and vice versa (Table 1). Importantly, Northern blot analysis confirmed that the water and glycerol transporter aquaporin 3 (AQP3) is regulated similarly to IGFBP3, such that AQP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53 (Fig. 9). Although AQP3 has not been linked to apoptosis to date, we cannot rule out the possibility that the increased expression of AQP3 may be proapoptotic by altering the water balance of the MCF7 cells.

DISCUSSION

How p53 differentially regulates target gene expression and thus chooses cell fate is complex. Previous studies have shown that the level of p53 (8, 48), the sequence of p53-responsive elements (47, 58), the proapoptotic AD2 (61, 70), the PRD (50, 69), and p53-associated proteins, such as ASPP1 and ASPP2 (33, 52), play an important role in this process. In this study, we have identified a common theme among the p53

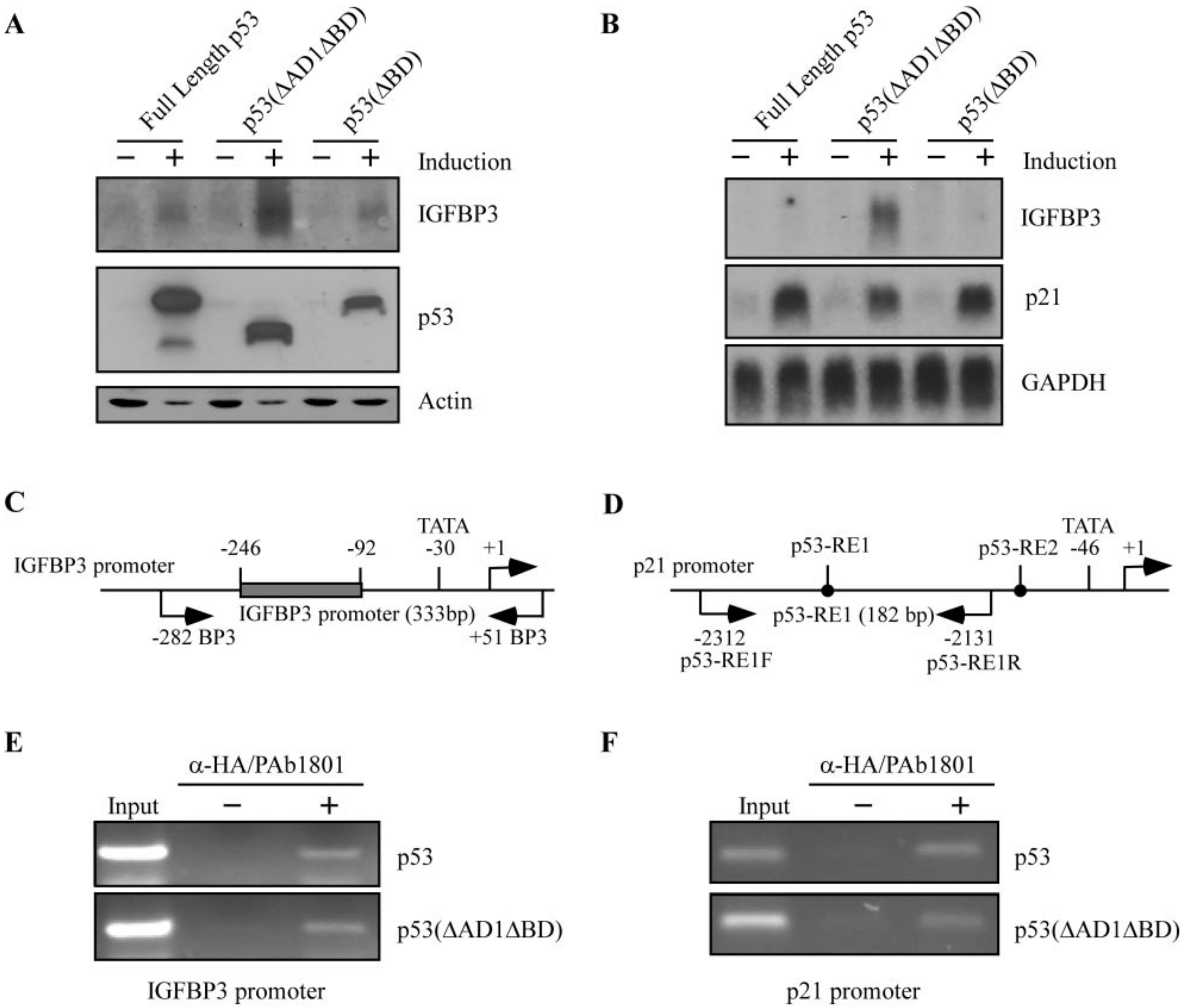

A
Full Length p53
p53(ΔAD1ΔBD)
p53(ΔBD)
− + − + − +
Induction
IGFBP3
p53
Actin
B
Full Length p53
p53(ΔAD1ΔBD)
p53(ΔBD)
− + − + − +
Induction
IGFBP3
p21
GAPDH
C
IGFBP3 promoter
-246
-92
TATA
-30
+1
-282 BP3
IGFBP3 promoter (333bp)
+51 BP3
D
p21 promoter
p53-RE1
p53-RE2
TATA
-46
+1
-2312
p53-RE1F
p53-RE1 (182 bp)
-2131
p53-RE1R
E
α-HA/PAb1801
Input
−
+
p53
p53(ΔAD1ΔBD)
IGFBP3 promoter
F
α-HA/PAb1801
Input
−
+
p53
p53(ΔAD1ΔBD)
p21 promoter

FIG. 7. IGFBP3 is induced by p53(ΔAD1ΔBD) but not by full-length p53 or p53(ΔBD). (A) Secreted IGFBP3 is induced by p53(ΔAD1ΔBD). Cell extracts and concentrated conditioned medium were prepared from cells uninduced (−) or induced (+) to express p53, p53(ΔAD1ΔBD), or p53(ΔBD). Levels of p53, actin, and secreted IGFBP3 in p53-24, p53(ΔAD1ΔBD)-15, and p53(ΔBD)-3 cell lines were determined by Western blotting with anti-p53 monoclonal antibodies, antiactin polyclonal antibody, and anti-IGFBP3 polyclonal antibody, respectively. (B) p53(ΔAD1ΔBD) transactivates the gene encoding IGFBP3. A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express p53, p53(ΔAD1ΔBD), or p53(ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes. (C and D) Schematic representation of the IGFBP3 and p21 promoters with the location of the transcriptional start site, the TATA box, p53-responsive elements, and primers used for ChIP assays. (E) p53 and p53(ΔAD1ΔBD) bind the IGFBP3 promoter in vivo. A ChIP assay was performed as described in Materials and Methods. (F) p53 and p53(ΔAD1ΔBD) bind the p53-responsive element within the p21 promoter in vivo.

family proteins: the C terminus is inhibitory, such that C-terminally truncated p53, p63, and p73 isoforms induce the expression of IGFBP3, an important effector of apoptosis, whereas full-length isoforms cannot. Thus, for the first time, we provide evidence that the BD of p53 is inhibitory in vivo as has been described in vitro. We have also shown that the in vivo inhibitory activity of the BD depends upon AD1, such that alleviation of inhibition by the BD requires deletion of AD1. We attribute the inhibition to HDAC activity. Inhibition of HDAC activity restores the ability of endogenous and exogenous full-length p53, p63, and p73 isoforms to induce IGFBP3. Moreover, inhibition of HDAC activity further enhances induction of IGFBP3 by C-terminally truncated p53 family isoforms (data not shown). We also identified several target genes differentially regulated by p53(ΔAD1ΔBD) and full-length p53, emphasizing the importance of p53 isoforms and functional domains for target gene selection. In summary, we have found that differential target gene selection requires the coordination of p53 family isoforms with the promoter environment.

p53 family isoforms differentially regulate target gene expression. Our studies indicate that coordinated and integrated signals are required for the induction of the proapoptotic target gene IGFBP3 by the p53 family. We showed that p53 functional domains regulate the ability of p53 to activate the IGFBP3 promoter, such that AD2 is required and AD1 and

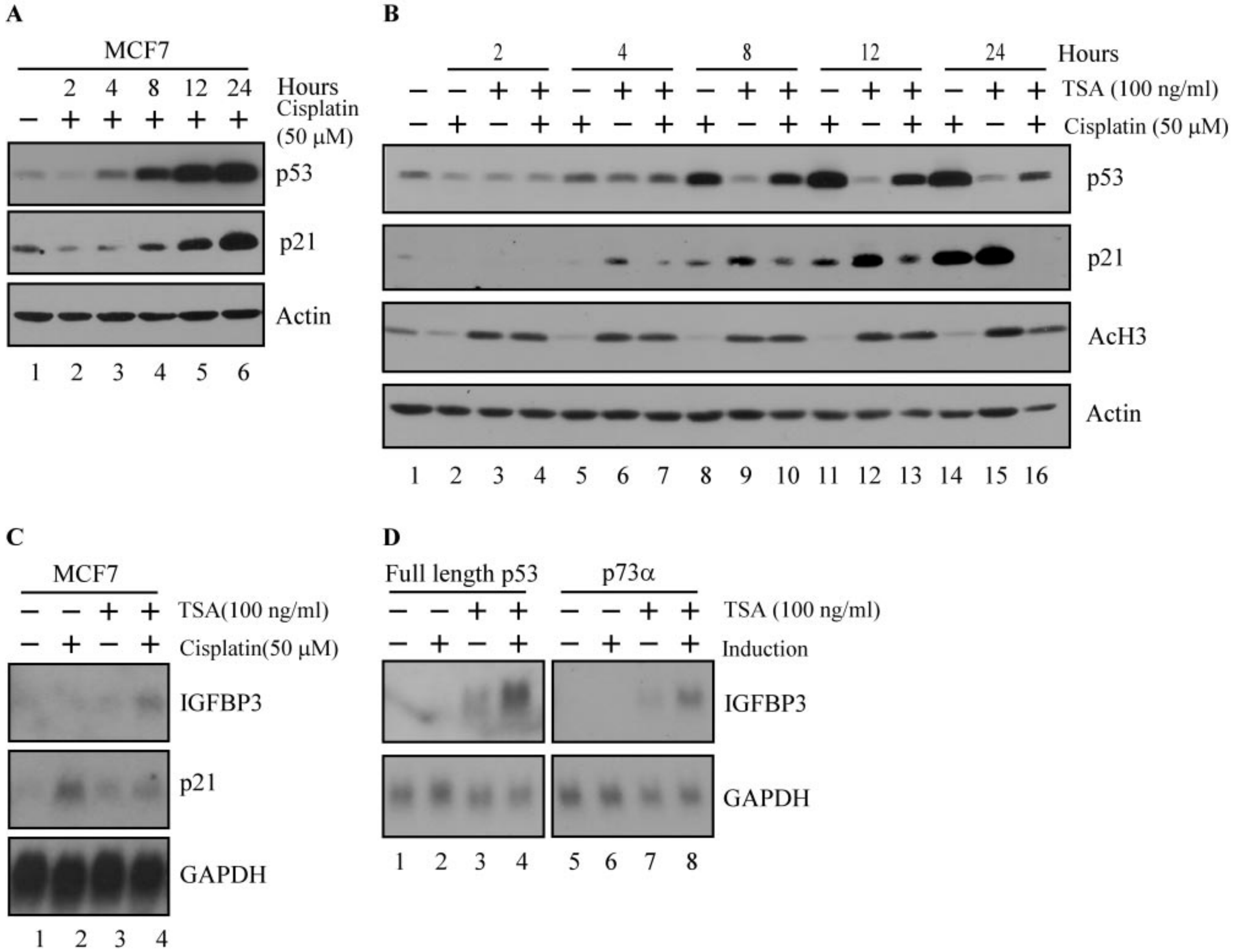


FIG. 8. HDAC activity inhibits the induction of IGFBP3 by full-length p53 family isoforms. (A) Time course of p53 stabilization by the DNA damage agent cisplatin. MCF7 cells were treated with 50 μM cisplatin, and cell extracts were collected at the time points indicated. p53, p21, and actin levels were determined by Western blotting by using anti-p53 monoclonal antibodies, anti-p21 polyclonal antibody, and antiactin polyclonal antibody, respectively. (B) Time course of p53 stabilization with cisplatin and/or HDAC inhibition with TSA. MCF7 cells were treated with 50 μM cisplatin and/or 100 ng of TSA per ml, and cell extracts were collected at the time points indicated. p53, acetylated histone H3, p21, and actin levels were determined by Western blotting by using anti-p53 monoclonal antibodies, anti-acetylated histone H3 polyclonal antibody, anti-p21 polyclonal antibody, and antiactin polyclonal antibody, respectively. (C) Inhibition of HDACs enables induction of IGFBP3 by endogenous p53. A Northern blot was prepared by using total RNAs isolated from MCF7 cells treated as indicated for 12 h. The blot was sequentially probed with cDNAs derived from IGFBP3, p21, and GAPDH genes. (D) Inhibition of HDACs restores the ability of exogenous p53 to induce IGFBP3. A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express p53 or p73α in the presence or absence of 100 ng of TSA per ml. The blot was sequentially probed with cDNAs derived from IGFBP3 and GAPDH genes.

the BD are inhibitory (Fig. 3). Interestingly, deletion of the inhibitory functional domains, namely the N-terminal AD1 and the C-terminal BD, is paralleled in nature. N-terminal truncation of p53 has been reported to occur (i) through alternative splicing of human p53 to yield ΔNp53/p47 (21); (ii) through the use of the internal translational start site at codon 40 and 41 in human and mouse p53, yielding ΔNp53 and Δ40p53, respectively (11, 12); (iii) through cleavage by calpain to yield a 42-kDa human and 41-kDa mouse p53 (31, 45); (iv) through interaction with mismatched double-stranded DNA to yield mouse p40(ΔN) (40); and (v) through interaction with an N- and C-terminally truncated p53 protein to yield mouse p50(ΔN23) (42). C-terminal truncation of p53 has been reported to occur (i) through alternative splicing to yield human I9+p53 and murine p53AS (1, 18); (ii) through cleavage by calpain (45); and (iii) through interaction with the 3′ end of ssDNA to yield murine p50(ΔC) and p40(ΔC) (42). Combined N- and C-terminal truncation has been reported to occur through interaction with mismatched DNA to yield p35, which is reported to have intrinsic protease ability (42), and through cleavage by calpain to yield a 33-kDa murine p53 (45). We postulate that combined N- and C-terminal truncation could occur sequentially. Alternative splicing or internal translational initiation at codon 40 generates ΔNp53, which is subsequently truncated at the C terminus through interaction with ssDNA or calpain. In the case of mouse p53, internal translational initiation of the p53AS transcript at codon 41 generates Δ40p53AS, which is analogous to p53(ΔAD1ΔBD). We found that both ΔNp53 and Δ40p53 behave similarly to p53(ΔAD1) (Fig. 4B and C). I9+p53 lacks the TD and, as expected, is

TABLE 1. Differential target gene regulation by full-length p53 and p53 (ΔAD1ΔBD)

Target gene	GenBank accession no.	Increase (fold) in expression of:	
		Full-length p53	p53 (ΔAD1ΔBD)
Common target gene			
p21	NM_000389.1	5.0	3.5
FDXR	NM_004110.2	5.1	3.2
Fas/Apo1	X83493.1	4.1	2.5
GADD45	NM_001924.2	3.6	2.5
CYFIP2	AL161999.1	2.3	2.0
TP53INP1	AW341649	3.2	3.4
TUBB	NM_001069.1	3.1	3.9
CAV2	NM_001233.1	3.4	3.8
Palladin	NM_016081.1	2.3	3.0
Differentially regulated gene			
AQP3	AB001325		3.2
EVA1	AF275945.1		4.6
MYO 10	NM_012334.1		2.2
Novel gene	AI188104		3.2
ANXA4	NM_001153.2	2.4	
MLF2	NM_005439.1	2.1	
POMZP3	NM_012230.1	3.0	

transcriptionally inactive (Fig. 4B). Interestingly, full-length mouse p53 and p53AS behave similarly to full-length p53 and human p53(ΔBD): mouse p53AS activates the IGFBP3 promoter, whereas full-length mouse p53 cannot (Fig. 4C). In addition, p53(ΔNΔBD), which mimics a sequentially truncated p53 isoform, is extremely active at the IGFBP3 promoter (Fig. 4B). Thus, for the first time, we showed that p53 isoforms do have different functions. In addition, using Affymetrix gene chip analysis, we identified several target genes differentially regulated by full-length p53 and p53(ΔAD1ΔBD) (Table 1).

Based on these data, we suggest a model according to which specific environmental stimuli trigger the generation of p53 isoforms that will differentially regulate target gene expression (Fig. 10A). Genotoxic stress induces DNA damage. The DNA damage pathway activates and stabilizes full-length p53, which in turn induces p53-dependent cell cycle arrest through induction of p21. The arrest allows a cell time to repair its damaged DNA. However, if the damage is extensive, free 3′ ends of the damaged DNA could induce N- and/or C-terminal cleavage of ΔNp53 and full-length p53 to yield p53(ΔNΔBD). Since p53 (ΔNΔBD) is resistant to negative regulation at the IGFBP3 promoter, IGFBP3 is induced. IGFBP3, along with other proapoptotic target genes, shifts the balance from p53-dependent cell cycle arrest to p53-dependent apoptosis. Thus, we propose that certain stimuli can alter the expression pattern of specific p53 isoforms and, subsequently, the expression pattern of p53 target genes (Fig. 10A). In support of this model, DNA damage has been shown to heighten the apoptotic response without affecting p53 levels (8). In addition, a recent study demonstrated that DNA damage-induced p53-dependent neuronal cell death was prevented upon calpain inhibition (54) and thus highlights the potential functional importance of calpain-dependent p53 isoforms.

Further strengthening the validity of our model is the evidence we found that p63 and p73 isoforms function in an analogous manner to p53 isoforms: the p63 and p73 C-terminally truncated isoforms, p63γ and p73β, but not full-length isoforms, induce the expression of IGFBP3 (Fig. 5C). Thus, the question arises, How are these isoforms differentially regulated? Since the regulation of alternative splicing has not been thoroughly studied, we propose a model where certain signals would activate alternative splicing to preferentially upregulate p63γ or p73β, which would carry out a specific transcriptional program, namely the induction of IGFBP3 and other proapoptotic target genes, and subsequently apoptosis (Fig. 10A). Therefore, future work needs to be done to characterize the regulation of alternative splicing.

Previously, we have shown that the ΔN isoforms of p63 and p73 contain a unique AD (13, 35) that differs from the AD in the TA isoforms. The AD in TAp63 and TAp73 shares 22 and 29% identity, respectively, to AD1 in p53 (Fig. 5A). Interestingly, we have shown that the p53 AD1 is inhibitory and AD2 is required for activation of the IGFBP3 promoter (Fig. 3B and C). Similarly, we found that the AD in the TA but not the ΔN isoforms is active in inducing IGFBP3 (Fig. 5C). Although the AD present in the TA isoforms is highly similar to AD1 in p53, perhaps the AD of TA possesses a wider range of functions, such that the critical residues or tertiary structure important for AD2 function are also contained within the TA AD. The fact that the ΔN isoforms cannot induce IGFBP3 suggests a functional difference between the ADs within the ΔN and TA isoforms.

The promoter environment contributes to differential target gene selection. Our studies indicate that the promoter sequence in terms of the structure and number of the p53-responsive elements and the promoter topology in terms of the associated proteins are important for target gene selection. The structure of the p53-responsive element within the

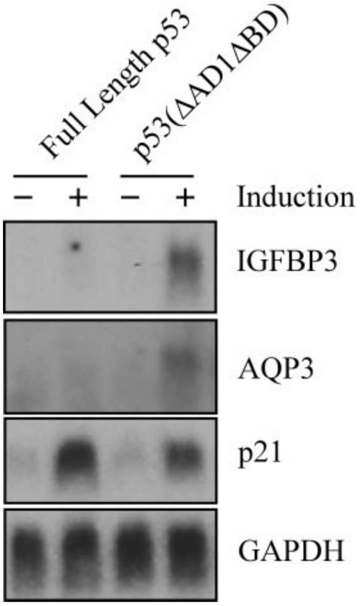


FIG. 9. Differential target gene regulation by full-length p53 and p53(ΔAD1ΔBD). A Northern blot was prepared by using total RNAs isolated from cells uninduced (−) or induced (+) to express p53 or p53 (ΔAD1ΔBD) for 24 h. The blot was sequentially probed with cDNAs derived from AQP3, IGFBP3, p21, and GAPDH genes.

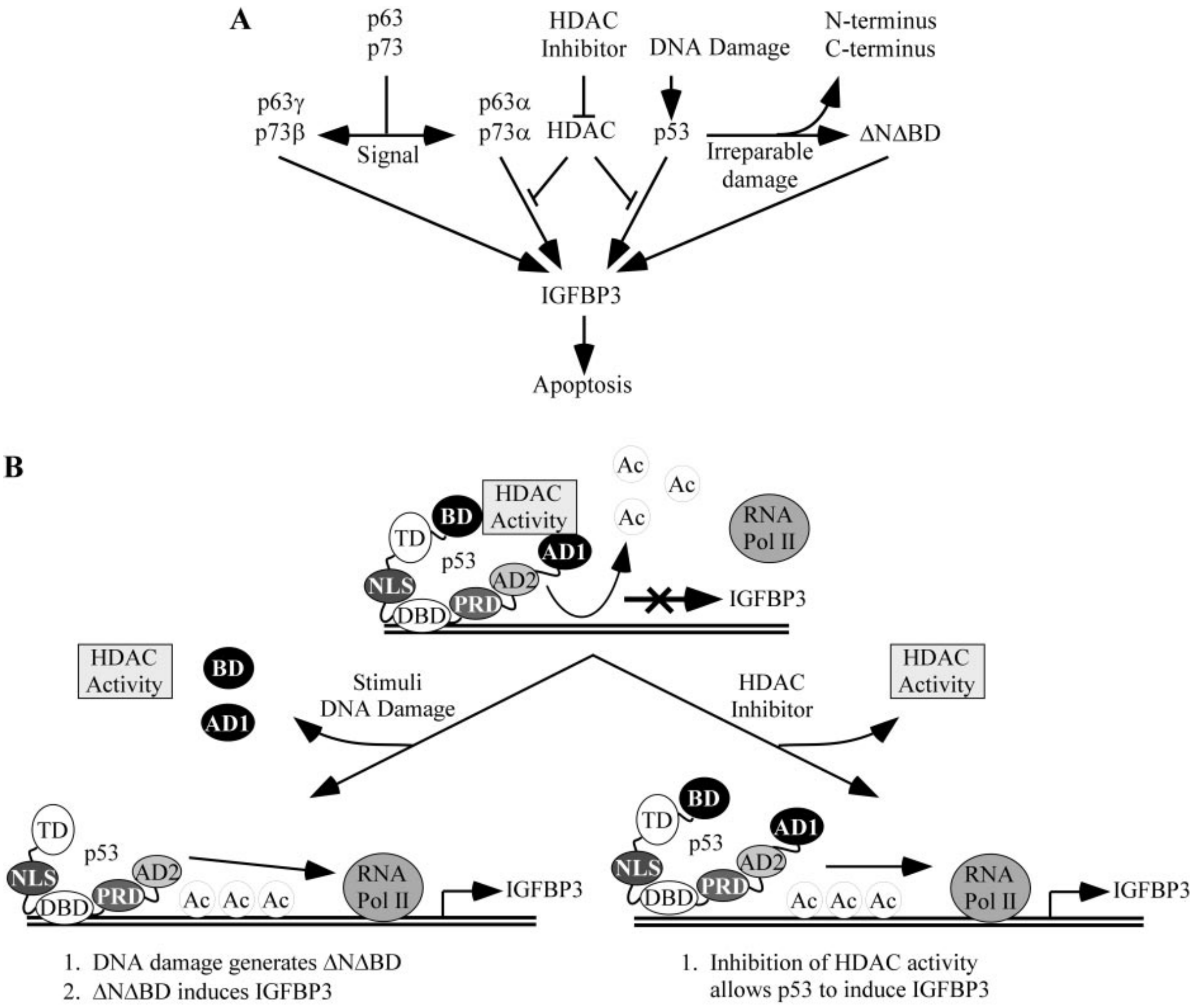

A
p63
p73
HDAC
Inhibitor
DNA Damage
N-terminus
C-terminus
p63γ
p73β
Signal
p63α
p73α
HDAC
p53
Irreparable
damage
ΔNΔBD
IGFBP3
Apoptosis
B
Ac
Ac
Ac
HDAC
Activity
BD
TD
p53
AD1
RNA
Pol II
NLS
AD2
PRD
DBD
IGFBP3
HDAC
Activity
BD
AD1
Stimuli
DNA Damage
HDAC
Inhibitor
HDAC
Activity
TD
p53
NLS
AD2
PRD
DBD
Ac Ac Ac
RNA
Pol II
IGFBP3
TD
BD
p53
AD1
NLS
AD2
PRD
DBD
Ac Ac Ac
RNA
Pol II
IGFBP3
1. DNA damage generates ΔNΔBD
2. ΔNΔBD induces IGFBP3
1. Inhibition of HDAC activity
allows p53 to induce IGFBP3

FIG. 10. A model of apoptosis for p53 family isoforms. Ac, acetyl.

IGFBP3 promoter is unique in that it contains 11 tandem decamers of the p53-responsive element. We found that the 11 decamers function as a unit, such that all are required for full activation of IGFBP3 by p53(ΔAD1ΔBD), p63γ, and p73β (Fig. 2E and 5F). Since only two decamers constitute the canonical p53-responsive element, these data suggest that the unique arrangement is important for the differential regulation of IGFBP3 by p53 family isoforms. Similar to IGFBP3, p53-induced gene 3 (PIG3) is a proapoptotic target gene and contains a unique p53-responsive element (46). p53 activates the PIG3 promoter through a pentanucleotide microsatellite sequence (10). Interestingly, the pentanucleotide sequence is polymorphic, and increasing numbers of repeats confer greater responsiveness to p53 (10). To further support the importance of the responsive element, studies show that p53-responsive elements located within cell cycle arrest genes are more highly activated by p53 than the ones located in proapoptotic genes (47). It has been postulated that the spacing between the decamers is critical. Responsive elements found within cell cycle arrest genes do not contain interspersed sequences between the decamers, but the ones within proapoptotic genes do (47, 58).

Our studies also demonstrate that the coordination of p53 family isoforms with the promoter environment is important for transcriptional activation of IGFBP3. We found that full-length p53 and p53(ΔAD1ΔBD) bind to the IGFBP3 promoter in vivo, as determined by the ChIP assay (Fig. 7E); however, only p53(ΔAD1ΔBD) is capable of inducing IGFBP3 (Fig. 1C). We found that inhibition of HDAC activity restores the ability of exogenous full-length p53 to induce IGFBP3 (Fig. 8D). In addition, we found that p63 and p73 isoforms behave in a similar fashion: HDAC activity inhibits the induction of IGFBP3 by full-length p63 and p73 isoforms, p63α and p73α, but not by C-terminally truncated isoforms, p63γ and p73β (Fig. 8D and data not shown). Our data suggest a model according to which full-length p53 and p53(ΔAD1ΔBD) bind to the IGFBP3 promoter, but full-length p53 cannot recruit the basal transcriptional machinery due to its association with HDAC activity (Fig. 10B). Although the acetylation status of p53 has been shown to modulate its own activity (27), our model focuses on the ability of p53 to interact either directly or indirectly with HDACs that target the chromatin but not full-length p53, as p53(ΔAD1ΔBD), which is extremely active in inducing IGFBP3, lacks acetylation sites. In support of our model, recent studies have demonstrated that p53 target gene expression requires more than just p53 binding: p53 first binds to the p21 promoter and recruits p300, which then acetylates the p21 proximal promoter and cooperates with p53 to induce p21 (16, 36).

In our model, p53 family isoforms differentially interact with regulatory proteins to facilitate target gene expression. Differential protein interactions may depend upon the conformation of the p53 protein or specific epitopes, either gained or lost. In support of the importance of conformation, many studies have identified p53 mutants that can induce apoptosis but not cell cycle arrest and vice versa (19, 49, 51). For example, mutant p53(121F) is a more potent inducer of transcription-dependent apoptosis than full-length p53 (51). Although the structure of p53(121F) was not determined by crystallography, because full-length p53 is a fluid molecule and will not crystallize, we speculate that the conformation of p53(121F) is different from that of the full-length p53. Thus, p53(121F) may escape regulation by certain proteins, potentially HDACs or protein complexes with HDAC activity, to enable the induction of proapoptotic target genes. Interestingly, p53 has been shown to interact with mSin3A, a component of the transcriptional repression complex, as well as HDAC-1, HDAC-2, and HDAC-3 (27, 71). While mSin3A interacts with the PRD of p53 (71), the physical interaction between p53 and HDAC-1, HDAC-2, and HDAC-3 has not been mapped. Thus, it is possible that the HDACs interact with the N and C termini of p53, and thus the IGFBP3 promoter bound by p53(ΔAD1ΔBD) escapes this negative regulation. While many questions still remain and many mechanisms are yet to be discovered, one point becomes clear: the ability of the p53 family to induce the expression of proapoptotic target genes is under stringent regulation.

Therapeutic implications. Restoration of p53 functions, especially p53-dependent apoptosis, is an attractive cancer therapeutic strategy. To exploit p53-dependent apoptosis for the destruction of cancer cells, many studies have focused on strategies that preferentially activate p53 to induce proapoptotic target genes. Here, we found that treatment of MCF7 cells with the DNA damage agent cisplatin was sufficient to stabilize p53 levels with the subsequent induction of p21; however, cisplatin alone was not sufficient to enable p53 induction of IGFBP3, an important effector of apoptosis. However, combined treatment with cisplatin and the HDAC inhibitor TSA enabled IGFBP3 expression (Fig. 8C). Thus, our data presented here shed new insight into how we can modulate p53 activity to induce apoptosis in tumor cells, which can be explored for the design of combined therapies for cancer treatment.

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Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity

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Abstract

Histone deacetylase (HDAC) inhibitors are emerging as promising cancer therapeutics. HDAC inhibitors have been found to induce cellular activities that are strikingly similar to p53-mediated responses to genotoxic stress. For example, HDAC inhibitors induce cell cycle arrest, apoptosis, and cellular senescence. Since at least eleven HDACs are affected by the current HDAC inhibitors, the HDAC critical for tumor cell survival and proliferation remains unknown. Thus, we sought to characterize the distinct roles of HDACs in the p53 pathway. Through the use of stable MCF7 cell lines which inducibly express shRNA targeting HDAC2, we found that HDAC2 plays important roles in the p53 pathway. Specifically, we found that knockdown of HDAC2 inhibited cellular proliferation in a dose-dependent manner which was also partly p53-dependent. Furthermore, knockdown of HDAC2 induced cellular senescence. Importantly, we found that knockdown of HDAC2 enhanced p53-dependent trans-repression and trans-activation of a subset of target genes. We found that the enhancement was due to increased p53-DNA binding activity but not alterations in p53 stability or post-translational modification(s). Thus, for the first time, our data suggest that HDAC inhibitors function through the p53 pathway, at least in part, by activating p53-DNA binding activity.

Introduction

The p53 tumor suppressor, a transcription factor, is well known to regulate target genes that mediate cell cycle arrest, apoptosis, senescence, DNA repair, and other responses to genotoxic stress (1, 2). The ability of p53 to regulate gene expression is under stringent control. The p53 protein is maintained at a low abundance in non-stressed cells by the E3 ubiquitin ligases Mdm2, Pirh2, and COP (3, 4). DNA damage-induced phosphorylation and acetylation of p53 promote the stabilization and transcriptional activities of p53 (5-7). Because p53 has the potential to induce many responses, we and others have investigated mechanisms of differential target gene regulation by p53. Through the use of histone deacetylase (HDAC) inhibitors, we have previously shown that HDACs play a role in differential target gene selection by p53 family proteins (8).

HDACs play important roles in many diverse processes such as transcriptional regulation, protein-protein interaction, protein sub-cellular localization, and organismal aging (9, 10). HDACs, which deacetylate histones and non-histone proteins, are organized into four classes: class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class III (SIRT 1-7), and class IV (HDAC 11) (9, 11). HDACs in classes I, II, and IV contain a conserved catalytic domain and are commonly inhibited by HDAC inhibitors such as Trichostatin A (TSA) and sodium butyrate (10). These inhibitors chelate the zinc cation within the enzyme active site (10). Class III HDACs, which contain an NAD-dependent catalytic domain, are insensitive to these agents (10). Recent evidence demonstrates that HDAC inhibitors induce apoptosis and inhibit proliferation in tumor cells (12-16). Although HDAC inhibitors are in phase I/II clinical trials and are emerging as promising cancer chemotherapeutics (9), the HDAC(s) critical for tumor cell survival and proliferation remains unknown.

p53 has recently been found to play an important role in mediating the effects of HDAC inhibitors. Upon HDAC inhibition, p53 has been found to be stabilized and acetylated at lysines 320, 373, and 382 (15-17). Although several lines of evidence suggest that HDAC inhibitors activate the p53 pathway, the role of p53 during HDAC inhibition and mechanisms by which individual HDACs regulate p53 activity remain unclear.

In this study, we sought to characterize the function of individual HDACs on p53 activity. Through the use of stable MCF7 cell lines which inducibly express shRNA targeting distinct HDACs, we found that knockdown of HDAC2, but not HDAC1, induced G1 arrest and inhibited cellular proliferation in a manner that partly depended on p53. Furthermore, knockdown of HDAC2 induced cellular senescence. Importantly, we found that knockdown of HDAC2 enhanced trans-repression and trans-activation of a subset of target genes by endogenous p53. Investigation into the mechanism revealed that knockdown of HDAC2 enhanced the ability of p53 to bind DNA *in vivo*. Thus, for the first time, we found that HDAC2 negatively regulates p53 activity by inhibiting p53-DNA binding.

Materials and Methods

Plasmids and reagents. HAp53/pcDNA3, p21A/pGL2, and pBabe-U6-p53-siRNA were as described (8, 18). To generate tetracycline-inducible Pol III driven expression of shRNA targeting HDAC1 or HDAC2, oligos were designed, annealed, and cloned into pTER vector. For

HDAC1 shRNA, oligos were HDAC1-si-CR-F: 5’GAT CCC C**GC AGA TGC AGA GAT TCA AC**T TCA AGA GA**G TTG AAT CTC TGC ATC TGC** TTT TTG GAA A-3’ and HDAC1-si-CR-R: 5’-AGC TTT TCC AAA AA**G CAG ATG CAG AGA TTC AAC** TCT CTT GAA **GTT GAA TCT CTG CAT CTG C**GG G-3’ with HDAC1 targeting sequence in bold. For HDAC2 shRNA, oligos were HDAC2-CR-644-F: 5’GAT CCC C**AA GCA TCA GGA TTC TGT TA**T TCA AGA GA**T AAC AGA ATC CTG ATG CTT** TTT TTG GAA A-3’ and HDAC2-CR-644-R: 5’-AGC TTT TCC AAA AA**A AGC ATC AGG ATT CTG TTA** TCT CTT GAA **TAA CAG AAT CCT GAT GCT T**GG G-3’ with HDAC2 targeting sequence in bold. Murine HDAC2 was subcloned from pME18s into pcDNA3 using EcoRI/XhoI. Reagents were tetracycline in EtOH at 2 µg/ml, doxycycline (Sigma) in H_2O at 1 µg/ml, nutlin-3 (Cayman Chemical Company, Ann Arbor, MI) in EtOH, *cis*-Diammineplatinum(II) dichloride (Sigma) in H_2O, and cyclohexamide (Sigma) in EtOH at 10 µg/ml. 21-bp annealed RNA oligos (HDAC2 siRNA: AAG CAU CAG GAU UCU GUU A and scrambled HDAC2 siRNA: GGC CGA UUG UCA AAU AAU U) were purchased from Dharmacon RNA Technologies.

Cell culture and transfection. MCF7, HCT116 p53$^{-/-}$, and MCF7-p53KD-3 cell lines were maintained as described (8, 18-20). Stable cell lines were generated as described (20). Individual clones were screened to identify MCF7 clone with stable integration of pcDNA6/TR (Invitrogen), named MCF7-TR7. Individual clones were screened to identify tetracycline-inducible knockdown of HDAC1 or HDAC2 by Western blot analysis, named MCF7-si-HDAC1-7, MCF7-si-HDAC2-10, MCF7-si-HDAC2-B18, and MCF7-si-HDAC2-B38. To generate MCF7 cell line with tetracycline-inducible knockdown of HDAC2 and constitutive knockdown of p53, pBabe-U6-p53-siRNA plasmid was transfected into MCF7-si-HDAC2-10 cells. Individual colonies were screened to identify clones with inducible knockdown of HDAC2 and constitutive knockdown of p53 by Western blot analysis, named MCF7-si-HDAC2-stable si-p53-13. For transient knockdown of HDAC2, MCF7 or MCF7-p53KD-3 (with stable p53-knockdown) cells were transfected with 50 nM oligos using siLentFect (BioRad) for 3d.

Western blot analysis. Western blot analysis was performed as described (20) using anti-p21 (C19; Santa Cruz), anti-Ac-p53(AcK373/382) (Upstate), anti-actin (Sigma), anti-HDAC1 (E210; Upstate), anti-HDAC2 (3F3; Upstate), anti-c-Myc (9E10.2), and anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies.

Growth rate, colony formation assay, and DNA histogram analysis. Growth rate was assayed as described (20). Briefly, $3x10^4$ cells were seeded with or without tetracycline in triplicate. Attached cells were counted at the indicated time. For colony formation assay, 500 cells were seeded with or without tetracycline in triplicate. Cells were fixed in methanol: glacial acetic acid (7:1), washed in H_2O, and stained with crystal violet (0.2 g/L). DNA histogram analysis was performed as described (20). Briefly, $1x10^5$ cells were seeded with or without tetracycline in triplicate, harvested, fixed in 100% ethanol, stained with propidium iodide, and analyzed by FACS.

Senescence-associated β-galactosidase assay. $1x10^3$ MCF7-si-HDAC2-10 cells were seeded with or without tetracycline in triplicate. Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS, washed in PBS, and stained overnight with 40 mM citric acid/sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-gal. % of SA-β-gal positive colonies were scored.

RNA isolation and Northern blot analysis. Total RNA was isolated using Trizol reagents (Invitrogen). Northern blot analysis was performed as described (8). The DKK1, GAPDH, Mdm2, p21, and FDXR probes were prepared as described (8, 20-22); the 338-bp c-Myc cDNA probe was amplified using primers as described (23) and cloned into pGEM-Teasy.

Luciferase assay. To determine the effect of HDAC2 knockdown on p53 activity, MCF7-si-HDAC2 cells were grown with or without tetracycline for 4d and co-transfected in triplicate with p21A/pGL2, internal control Renilla luciferase vector pRL/CMV, and either empty pcDNA3 or pcDNA3 expressing HA-tagged wild-type p53 for 24h. To determine the effect of HDAC2 on p53 activity, HCT116 $p53^{-/-}$ and MCF7 cells were co-transfected in triplicate with p21A/pGL2, pRL/CMV, and an equal concentration of pcDNA3, p53, and HDAC2 expression vectors as indicated for 24h. The dual luciferase reporter assay (Promega) was performed. Fold increase in luciferase activity is shown. Standard error was determined.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed as described (8). Briefly, MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3d and treated with 5 µM nutlin or 50 µM cisplatin as indicated. Chromatin was crosslinked and sonicated. DNA was quantitated and equal amounts of chromatin were immunoprecipitated with anti-mSin3A (K20; Santa Cruz), anti-AcH3 (Upstate), anti-p53 DO-1, or a mix of anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies. Control antibodies were non-immune rabbit IgG

(Sigma), mouse IgG2a (UPC-10; Sigma), and anti-mouse (Sigma). Immunoprecipitated DNA fragments were purified over a QIAGEN column and analyzed by PCR. Primers that amplify the GAPDH promoter and the 5’ p53-responsive elements within the p21 and Mdm2 promoters were as described (8, 24, 25). Primers that amplify a 208bp fragment spanning the p53-responsive element within the human c-Myc gene were the 5’end primer Myc-1765-5’ (TGA GGG ACC AAG GAT GAG AAG AAT G) and 3’ end primer Myc-1104-3’ (TGA AAG TGC ACT GTA TGT AAC CCG C). Primers that amplify a 189bp fragment spanning the +1 site of the DKK1 gene were the 5’ end primer DK1-2996F (CAG TCA GGA CTC TGG GAC CGC AGG G) and the 3’ end primer DK1-3185R (GCC GCT ACC ATC GCG ACA AAG ACC C).

Affymetrix gene chip analysis. Total RNAs from MCF7-si-HDAC2-10 cells grown with or without tetracycline for 4d were isolated, labeled, and hybridized to an Affymetrix gene chip (U133 plus 2.0).

Results

Knockdown of HDAC2, but not HDAC1, inhibits cellular proliferation and induces cellular senescence

To characterize the role of individual HDACs on p53 activity, we generated stable MCF7 cell lines which inducibly express shRNA targeting HDAC1 or HDAC2 in the tetracycline-inducible system named MCF7-si-HDAC1 clone 7 and MCF7-si-HDAC2 clone 10, respectively.

Western blot analysis demonstrated that HDAC1 and HDAC2 protein levels were significantly decreased upon expression of shRNA targeting HDAC1 and HDAC2, respectively (Fig. 1A). Interestingly, we found that knockdown of HDAC2, but not HDAC1, resulted in a G1 arrest (Fig. 1B). Consistent with the arrest in G1, cellular proliferation (Fig. 1C) and colony formation (Fig. 1D) were inhibited upon knockdown of HDAC2. We also found that HDAC2 affected colony formation in a dose-dependent manner, since more effective knockdown of HDAC2 resulted in more marked growth inhibition (Fig. 1D). In contrast, knockdown of HDAC1 or tetracycline had no effect on cellular proliferation or colony formation (Fig. 1C and D). Furthermore, we found that knockdown of HDAC2 resulted in cellular senescence (Fig. 1E). Thus, HDAC2 is required for cellular proliferation and is critical for the inhibition of cellular senescence.

Because p53 is well known to inhibit cellular proliferation (1), we sought to determine if p53 was responsible for the growth arrest induced upon knockdown of HDAC2. Thus, we generated cell lines which inducibly express shRNA targeting HDAC2 and constitutively express shRNA targeting p53. Western blot analysis demonstrated that p53 and HDAC2 protein levels were constitutively and inducibly down-regulated, respectively, in a representative cell line named MCF7-si-HDAC2-stable si-p53 clone 13 (Fig. 2A). Consistent with the role of p53 in growth arrest, we found that the proliferative defect induced upon knockdown of HDAC2 was partly rescued by knockdown of p53 as measured by cellular proliferation (Fig. 2B) and colony formation (Fig. 2C) assays. Thus, knockdown of HDAC2 inhibited proliferation in both p53-dependent and -independent manners. In sum, the inhibition of proliferation and the induction of cellular senescence upon knockdown of HDAC2 suggest that HDAC2 plays an important role in the promotion of tumorigenesis, in part through the inhibition of p53 activity, and that HDAC2 is a critical target of HDAC inhibitors for cancer therapy.

HDAC2 does not affect p53 stability

Since we have found that knockdown of HDAC2 inhibited proliferation in a manner that partly depended on p53, we sought to determine the mechanism by which HDAC2 inhibited p53 activity. Since HDAC inhibitors have been found to induce the stabilization of p53 (15-17), we wanted to determine if knockdown of HDAC1 or HDAC2 affected p53 stability. We found that knockdown of HDAC1 or HDAC2 did not affect the stabilization of p53 in cells treated with the chemotherapeutic agent cisplatin or the Mdm2-inhibitor nutlin (Fig. 3A; also see 5B). Although a small increase in the abundance of p53 was detected upon knockdown of HDAC2 together with cisplatin treatment, this increase was not consistently observed and was not detected upon knockdown of HDAC2 together with nutlin treatment (Fig. 3A, compare lanes 9-10 with 11-12, respectively). Furthermore, knockdown of HDAC2 did not enhance cisplatin-induced acetylation of p53 as measured by Western blot analysis of whole cell lysates or p53 immunoprecipitates with anti-acetyl-p53(K373/K382) antibody or anti-acetyl lysine antibody, respectively (Fig. 3A and Supplementary Fig. 1). This is consistent with evidence that p53 can be deacetylated by HDAC1 and HDAC3, but not by HDAC2 (26-28). In line with this, knockdown of HDAC2 did not prolong the half-life of p53 (Fig. 3B). Taken together, these data suggest that HDAC2 alone does not greatly affect p53 stabilization in response to cisplatin or nutlin.

HDAC2 inhibits p53-dependent trans-repression of *c-Myc*

Since knockdown of HDAC2 did not affect p53 stabilization or acetylation, we sought to determine if the expression of p53 target genes was affected upon knockdown of HDAC2. We found that p53 repressed genes, *c-Myc* and *Cyclin B1*, were down-regulated upon knockdown of HDAC2 (Fig. 4 and Supplementary Fig. 2). We found that c-Myc mRNA was reduced upon knockdown of HDAC2, but not HDAC1 (Fig. 4A). The abundance of c-Myc protein was also reduced upon inducible as well as transient knockdown of HDAC2 (Fig. 4B and C). This is consistent with reports that *c-Myc* is repressed by HDAC inhibitors (29-31). Therefore, for the first time, we have identified that knockdown of HDAC2, but not HDAC1, results in the repression of *c-Myc* (Fig. 4A and B). Since p53 is known to directly repress *c-Myc* (32), we sought to determine the role of p53 in the repression of *c-Myc* upon knockdown of HDAC2. To do this, we transiently knocked-down HDAC2 in MCF7 cells and in MCF7 cells in which p53 was stably knocked-down. We found that the repression of *c-Myc* upon knockdown of HDAC2 was partly dependent on p53 (Fig. 4C, compare lanes 1 and 3 with 2 and 4, respectively). To investigate the mechanism, we characterized the binding of p53 to the *c-Myc* promoter by chromatin immunoprecipitation (ChIP) analysis and found that in the unstressed condition, the extent of p53 bound to the p53-responsive element within the *c-Myc* promoter was markedly increased upon knockdown of HDAC2 (Fig. 4D). mSin3A, a component of the p53-repression complex, was also found to interact with the *c-Myc* promoter, but the interaction was not affected by HDAC2 knockdown (Fig. 4D). Consistent with the repressed state of the *c-Myc* promoter, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 (Fig. 4D). Immunoprecipitation with rabbit-IgG served as a negative control in the ChIP assay (Fig. 4D).

Thus, HDAC2 is required for the basal expression of c-Myc by inhibiting p53-dependent repression of c-Myc.

HDAC2 inhibits p53-dependent trans-activation of a subset of target genes

Because HDAC2 inhibited p53-dependent trans-repression of *c-Myc*, we investigated whether HDAC2 could also affect p53-dependent trans-activation. First, we sought to determine if HDAC2 inhibited *IGFBP3* or *AQP3* induction by p53, since HDAC inhibitors restore the ability of endogenous p53 to induce *IGFBP3* and possibly *AQP3* in MCF7 cells (8). To test this, HDAC2 was inducibly knocked-down in cells treated with or without cisplatin or nutlin. We found that knockdown of HDAC2 was not sufficient to activate endogenous p53 to induce *IGFBP3* or *AQP3* in MCF7 cells (data not shown). Thus, a combination of HDAC2 and another HDAC may be involved in repressing endogenous p53 to induce *IGFBP3* and *AQP3*.

To identify other genes regulated by HDAC2, we performed a microarray study. Although 2% of genes can be affected upon HDAC inhibition (33), only a small number of genes, such as *Dickkopf-1 (DKK1)* (NM_012242.1), *DUSP5* (NM_004419.2), and *Claudin-1 (CLDN1)* (AF101051.1), were found to be affected ≥ 2-fold upon knockdown of HDAC2. As an internal control, the expression of *HDAC2* was found to be decreased by 5.3-fold upon knockdown of HDAC2. Since we have previously found *DKK1* to be a p53 target gene (21), we chose *DKK1* for further analysis. Indeed, knockdown of HDAC2 led to a modest *DKK1* induction (Fig. 5A). Strikingly, we found that knockdown of HDAC2 together with cisplatin or nutlin treatment led to a robust *DKK1* induction whereas treatment with cisplatin or nutlin alone led to a modest *DKK1* induction (Fig. 5A). The induction of the *p21, Mdm2,* and *FDXR* genes by p53 was also further enhanced upon knockdown of HDAC2 (Fig. 5A). Thus, HDAC2 regulates p53 transcriptional activity, at least for a subset of p53 target genes.

To further characterize the role of HDAC2 in the p53 pathway, we examined the effect of HDAC2 on *p21* induction by p53. Consistent with the level of p21 mRNA, p21 protein was induced to a greater extent upon knockdown of HDAC2, either inducibly in MCF7 cells or transiently in RKO colon carcinoma cells, together with nutlin treatment compared to nutlin treatment alone (Fig. 5B). We also found that knockdown of HDAC2 led to a modest activation of the p21 promoter and that knockdown of HDAC2 further enhanced the activation of the p21 promoter by p53 (Fig. 5C). Conversely, overexpression of HDAC2 repressed the ability of p53 to activate the p21 promoter in HCT116-p53$^{-/-}$ and MCF7 cells (Fig. 5D). Taken together, HDAC2 inhibits the induction of p21 by p53.

HDAC2 inhibits p53-DNA binding activity

Since knockdown of HDAC2 did not alter the stability or acetylation of p53 (Fig. 3), enhanced p53 transcriptional activities upon knockdown of HDAC2 are not likely due to p53 abundance. Because knockdown of HDAC2 enhanced p53-DNA binding at the *c-Myc* promoter (Fig. 4D), we investigated whether HDAC2 could also affect p53-DNA binding activity at p53-induced genes by ChIP analysis. Indeed, we found that knockdown of HDAC2 together with nutlin or cisplatin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the *p21* promoter when compared to p53-DNA binding upon nutlin or cisplatin treatment alone (Fig. 6A, 6B, & Supplementary Fig. 3A). Although the cisplatin-induced p53-

p300 interaction was not greatly altered upon knockdown of HDAC2 (data not shown), the increase in p53-DNA binding was associated with a concomitant increase in binding of the co-activator p300 to the p21 promoter (Supplementary Fig. 3B). Furthermore, knockdown of HDAC2 together with nutlin or cisplatin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the *Mdm2* promoter and to a putative p53-responsive element near the transcriptional start site of the *DKK1* gene (Fig. 6C). Anti-mouse and mouse IgG2a antibodies served as negative controls in the ChIP assay (Fig. 6). As an additional control, p53 was not found to bind the *GAPDH* promoter (Fig. 6C)

While it is well accepted that the binding of an activator facilitates the hyper-acetylation of core histones at some promoters (34), surprisingly, we found that the level of histone H3 acetylation did not parallel the increase in binding of p53 and p300 to the *p21* promoter. When compared to the unstressed condition, we found that the level of histone H3 acetylation increased upon treatment with nutlin or cisplatin regardless of HDAC2 status (Fig. 6A & Supplementary Fig. 3A). However, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 together with nutlin or cisplatin treatment compared to nutlin or cisplatin treatment alone (Fig. 6A & Supplementary Fig. 3A). Thus, knockdown of HDAC2 together with cisplatin or nutlin treatment correlated with increased p53- and p300-binding and decreased histone H3 acetylation at the p21 promoter. This suggests that a corepressor, such as HDAC2, exerts a unique impact on the promoter for gene regulation.

Discussion

HDAC inhibitors, which have been shown to inhibit proliferation and to induce apoptosis and cellular senescence (13-15, 35, 36), are emerging as promising cancer therapies (9). Here we have identified that HDAC2 is a critical target for HDAC inhibition. We showed that inducible knockdown of HDAC2 induced G1 arrest, inhibited cellular proliferation, and induced cellular senescence. Our data is consistent with recent studies which have shown that transient knockdown of HDAC2 inhibits proliferation and induces apoptosis (37, 38). Importantly, we found that the proliferative defect induced upon knockdown of HDAC2 was partly p53-dependent. In addition, we showed that knockdown of HDAC2 lead to the repression of *c-Myc* that was partly dependent on p53. Since *c-Myc* is required for cellular proliferation in some cell types (39-41), it is likely that the repression of *c-Myc* plays an important role in the proliferation defect upon knockdown of HDAC2. Furthermore, through our microarray study, we identified and confirmed that *claudin-1 (CLDN1)*, a component of the tight junction, was induced upon knockdown of HDAC2 (Supplementary Fig. 4A & B). Since *CLDN1* expression has been found to be increased in senescent compared to proliferating mammary epithelial cells (42), perhaps *CLDN1* plays a role in the senescence induced upon knockdown of HDAC2. Taken together, these data suggest that HDAC2 promotes cellular proliferation and prevents cellular senescence and is thus a critical target of HDAC inhibitors.

The function of HDAC2 as a co-repressor is well established. HDAC2 plays an important role in transcriptional repression by the mSin3a and NURD complexes as well as by transcription factors such as Mad, YY1, p53, and others (27, 43-46). In this study, we have found that HDAC2 negatively regulates p53 transcriptional activities such that HDAC2 not only inhibits the ability of p53 to trans-activate but also to trans-repress a subset of genes. We showed that knockdown of HDAC2 enhanced p53 induction of *p21*, *DKK1*, *Mdm2*, and *FDXR*, but not *IGFBP3* or *AQP3*. Furthermore, knockdown of HDAC2 repressed *c-Myc* and *Cyclin B1*. Investigation into the mechanism revealed that enhanced trans-activation and trans-repression by p53 was due to augmented p53-DNA binding activity but not alterations in p53 stability or acetylation.

The precise mechanism by which HDAC2 inhibits p53-DNA binding remains unclear. Several hypotheses exist: (i) HDAC2 directly inhibits p53-DNA binding activity, (ii) HDAC2 deacetylates p53 at unknown lysine residue(s) to inhibit p53-DNA binding activity, or (iii) HDAC2 modulates chromatin structure which affects p53-DNA binding activity. Since HDAC2 has been found to directly interact with p53 (27), the first hypothesis is formally possible. Evidence suggests that acetylation of C-terminal lysine residues in p53 enhances p53-DNA binding activity *in vitro* and *in vivo* (47-49). Although cisplatin-induced acetylation of lysines 373 and 382 was not altered upon knockdown of HDAC2, HDAC2 may act on the eighteen other lysine residues within p53. Thus, the second hypothesis is formally possible. However, several lines of evidence suggest that p53 acetylation and stabilization are linked (6, 28). Since p53 stability was not affected upon knockdown of HDAC2, p53 acetylation may not be responsible for the augmented p53-DNA binding activity. Unexpectedly, we found that the level of histone H3 acetylation was diminished upon knockdown of HDAC2. The decrease in histone H3 acetylation correlated with the increase in p53-DNA binding activity. Thus, our data may support the third hypothesis where HDAC2, although a deacetylase, impacts p53-DNA binding through the modulation of histone acetylation and chromatin composition. In line with this, HDAC2 has been shown to interact with topoisomerase II which plays an important role in chromatin structure (45). Furthermore, *Myc* has recently been identified to influence global chromatin structure whereby lack of *Myc* led to a striking decrease in overall histone acetylation (50). Perhaps knockdown of HDAC2 induces an initial p53-independent repression of *c-Myc* which then triggers a feedforward loop of decreased histone H3 acetylation and increased p53-DNA binding activity. Regardless, the reduced acetylation of histone H3 may enhance the electrostatic interactions between p53 and chromatin, thereby stabilizing the p53-DNA interaction.

In summary, we showed that knockdown of HDAC2 inhibited cellular proliferation in a manner that partly depended on p53, induced cellular senescence, and augmented p53-dependent trans-activation and trans-repression of a subset of target genes. This is the first report, to our knowledge, to demonstrate that HDAC2 negatively regulates p53-DNA binding activity. Future studies need to address the precise mechanism by which HDAC2 inhibits p53-DNA binding as well as whether HDAC2 affects the DNA binding activity of mutant p53 or other p53 family proteins. Since the expression of HDAC2 has been found to be increased in cancers (37, 38), our study suggests that HDAC2 is a critical target of HDAC inhibitors in cancer therapies.

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Figure Legends

Figure 1. Knockdown of HDAC2, but not HDAC1, induces G1 cell cycle arrest, inhibits cellular proliferation, and induces cellular senescence. (A) The abundance of HDAC1 and HDAC2 is reduced in stable MCF7 cell lines upon tetracycline-inducible expression of shRNA targeting HDAC1 and HDAC2, respectively. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis with antibodies against HDAC1, HDAC2, and actin, respectively, in MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d. (B) Knockdown of HDAC2, but not HDAC1, induces G1 arrest. The percentages of MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells in G1, S, and G2/M phases grown with or without tetracycline for 4d were determined by DNA histogram analysis. (C) Knockdown of HDAC2 inhibits cell proliferation. Growth rates for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2-10 cells grown with or without tetracycline were measured by Coulter cell counter. Error bars denote standard deviation. (D) Colony formation assay for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2 clones B18, B38, and 10. Cells were grown with or without tetracycline for 12d, fixed, and stained with crystal violet. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis in MCF7-si-HDAC2 clones B18, B38, and 10 grown with or without tetracycline for 3d. (E) Knockdown of HDAC2 induces cellular senescence. MCF7-si-HDAC2-10 cells were grown with or without tetracycline, fixed, and stained for senescence-associated β-gal activity at the indicated time. Phase contrast microscopy of representative colonies (E, Upper) and quantitation of SA-β-gal positive colonies (E, Lower) are shown. Error bars denote standard error.

Figure 2. Knockdown of HDAC2 inhibits cellular proliferation in p53-dependent and -independent manners. (A) MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells were grown with or without tetracycline for 3d. The level of HDAC2, p53, and actin was assayed by Western blot analysis. (B) Stable knockdown of p53 partly rescues growth arrest induced upon knockdown of HDAC2. MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells were grown with or without tetracycline for 6d, collected, and counted by Coulter cell counter. Bars represent an average of three independent experiments each performed in triplicate. Error bars denote standard deviation. (C) Colony formation assay for MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells. Cells were grown with or without tetracycline for 15d, fixed, and stained with crystal violet.

Figure 3. Knockdown of HDAC1 or HDAC2 does not affect p53 stability. (A) MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3d and treated with 50 µM cisplatin or 5 µM nutlin for 13h. The level of HDAC1, HDAC2, p53, p53-AcK373/382, and actin was assayed by Western blot analysis. (B) Knockdown of HDAC2 does not prolong the half-life of p53. MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3d and treated with cyclohexamide (CHX) for the indicated time. The level of p53 and actin was assayed by Western blot analysis.

Figure 4. Knockdown of HDAC2 represses c-Myc in a p53-dependent manner. (A) Knockdown of HDAC2, but not HDAC1, represses c-Myc mRNA. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d. The blot was sequentially probed with cDNAs

derived from *c-Myc* and *GAPDH* genes. (B) Knockdown of HDAC2 represses c-Myc protein. The level of HDAC1, HDAC2, c-Myc, and actin was assayed by Western blot analysis in MCF7 parental and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d. (C) Repression of c-Myc upon knockdown of HDAC2 is partially p53-dependent. Parental MCF7 cells and MCF7 cells in which p53 was stably knocked-down (MCF7-p53KD-3) were transiently transfected with scrambled or HDAC2 siRNA oligos for 3d. The level of HDAC2, c-Myc, p53, and actin was assayed by Western blot analysis. s.e. and l.e. denote short and long exposure, respectively. (D) Knockdown of HDAC2 enhances p53-DNA binding to the p53-responsive element within the *c-Myc* promoter. (D, Upper) Schematic representation of the human *c-Myc* promoter and location of transcriptional start sites, p53-responsive element, and primers used for ChIP assay. Lower case italicized nucleotides identify mismatches from p53 consensus binding site. (D, Lower left) ChIP assay was performed using MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d. Chromatin was immunoprecipitated with p53, mSin3A, and control rabbit IgG antibodies. The fragment containing the p53-responsive element in the *c-Myc* promoter was amplified by PCR. (D, Lower right) ChIP assay was performed using Ac-histone H3 and control rabbit IgG antibodies.

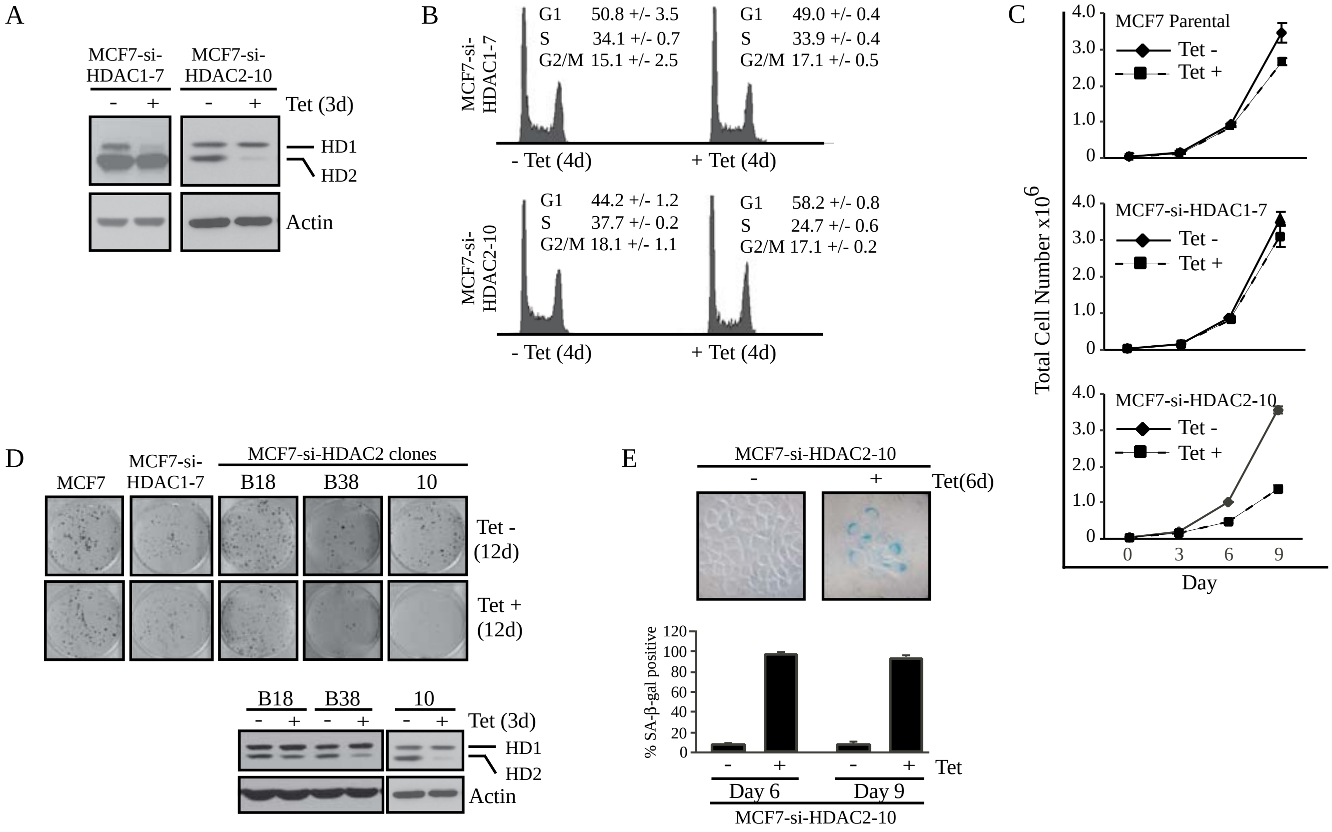
Figure 5. Knockdown of HDAC2 enhances p53 transcriptional activity for a subset of target genes. (A) Knockdown of HDAC2 enhances p53 activity to induce *DKK1*, *p21*, *Mdm2*, and *FDXR* genes. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d and treated with 50 µM cisplatin or 5 µM nutlin as indicated. The blots were probed with cDNAs derived from the *DKK1*, *p21*, *Mdm2*, *FDXR*, and *GAPDH* genes. (B) Knockdown of HDAC2 enhances the induction of p21

by p53 in MCF7 and RKO cells. The level of HDAC1, HDAC2, p53, p21, and actin was assayed by Western blot analysis in MCF7-si-HDAC2-10 cells (B, Left) treated as indicated and in RKO cells (B, Right) transiently transfected with scrambled oligos or HDAC2 siRNA oligos along with treatment of nutlin as indicated. (C) Knockdown of HDAC2 activates the p21 promoter and further enhances activation of the p21 promoter by p53. (C, Upper) Schematic representation of the p21 luciferase reporter with p53-responsive elements and TATA box shown. (C, Lower) MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 4d and transfected with the p21 luciferase reporter along with an empty pcDNA3 or pcDNA3 expressing p53 as indicated for 24h. Fold increase in luciferase activity is shown. (D) HDAC2 diminishes the activation of the p21 promoter by p53. HCT116 p53$^{-/-}$ (D, Left) or MCF7 (D, Right) cells were transfected with the p21 luciferase reporter along with pcDNA3, HDAC2, or p53 expression vector as indicated for 24h. Fold increase in luciferase activity is shown. Error bars denote standard error.

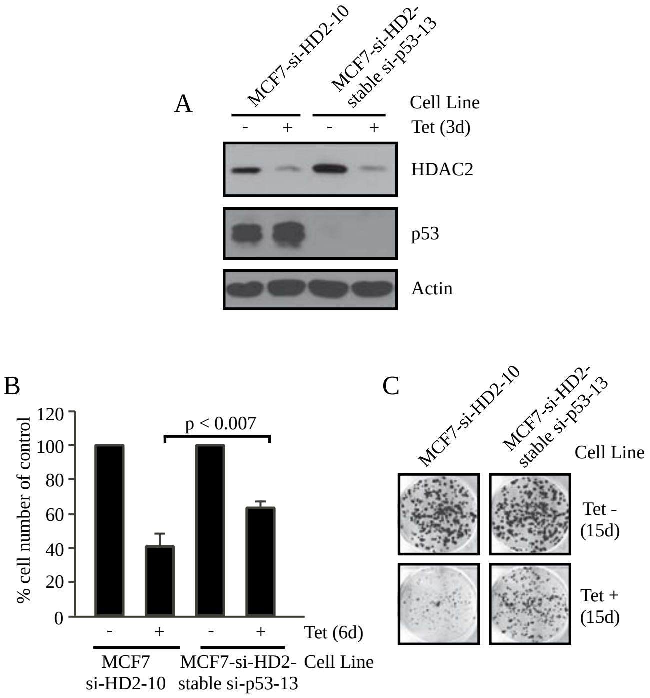
Figure 6. HDAC2 inhibits p53-DNA binding. (A and B) Knockdown of HDAC2 enhances p53-DNA binding at the endogenous p21 promoter upon treatment with nutlin (A) and cisplatin (B). ChIP assay was performed using MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d and treated with 5 µM nutlin (A) or 50 µM cisplatin (B) as indicated. Chromatin was immunoprecipitated with anti-p53 (either a mixture of DO1, PAb-1801, PAb-240, and PAb-421 or DO1 alone), anti-Ac-histone H3, control anti-mouse, or control mouse IgG2a antibodies as indicated. p53-RE1 within the *p21* gene was amplified by PCR. (C) Knockdown of HDAC2 enhances p53-DNA binding at *DKK1* and *Mdm2* genes. ChIP assay was performed as described above. A potential p53 responsive element near the transcriptional start

site of the *DKK1* gene and p53-RE1 within the *Mdm2* gene were amplified by PCR. The GAPDH promoter was amplified and served as an additional negative control for p53-DNA binding.

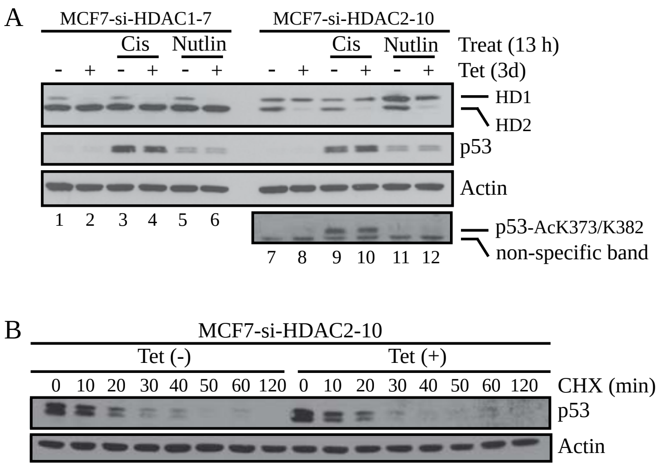
Harms and Chen - Figure 1



Harms and Chen - Figure 2



Harms and Chen - Figure 3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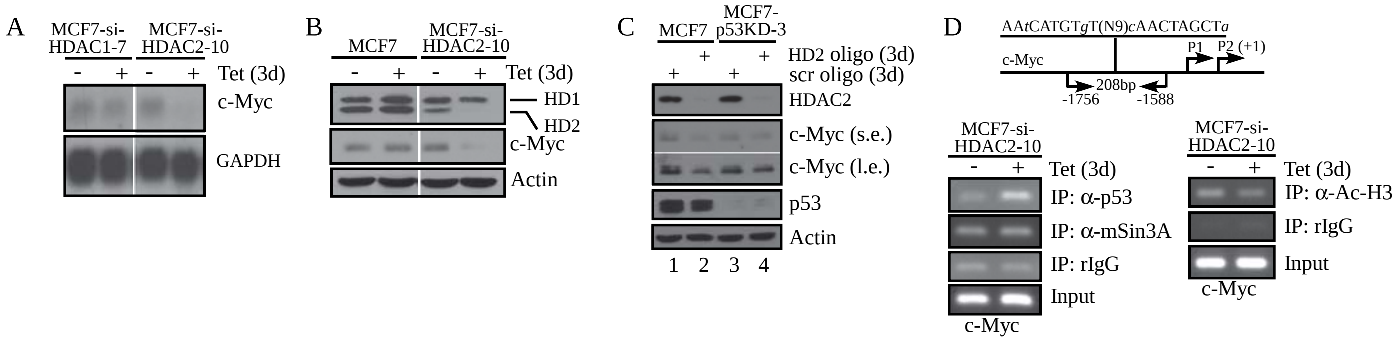
MCF7-si-HDAC2-10
Cis
Nutlin
Cis
Nutlin
Treat (13 h)
- + - + - + - + - + - +
Tet (3d)
HD1
HD2
p53
Actin
1 2 3 4 5 6
p53-AcK373/K382
non-specific band
7 8 9 10 11 12
B
MCF7-si-HDAC2-10
Tet (-)
Tet (+)
0 10 20 30 40 50 60 120 0 10 20 30 40 50 60 120
CHX (min)
p53
Actin

Harms and Chen - Figure 4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MCF7-si-HDAC2-10
- + - +
Tet (3d)
c-Myc
GAPDH
B
MCF7
MCF7-si-HDAC2-10
- + - +
Tet (3d)
HD1
HD2
c-Myc
Actin
C
MCF7
MCF7-p53KD-3
+ +
HD2 oligo (3d)
+ +
scr oligo (3d)
HDAC2
c-Myc (s.e.)
c-Myc (l.e.)
p53
Actin
1 2 3 4
D
AAtCATGTgT(N9)cAACTAGCTa
c-Myc
P1
P2 (+1)
208bp
-1756
-1588
MCF7-si-HDAC2-10
- +
Tet (3d)
IP: α-p53
IP: α-mSin3A
IP: rIgG
Input
c-Myc
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- +
Tet (3d)
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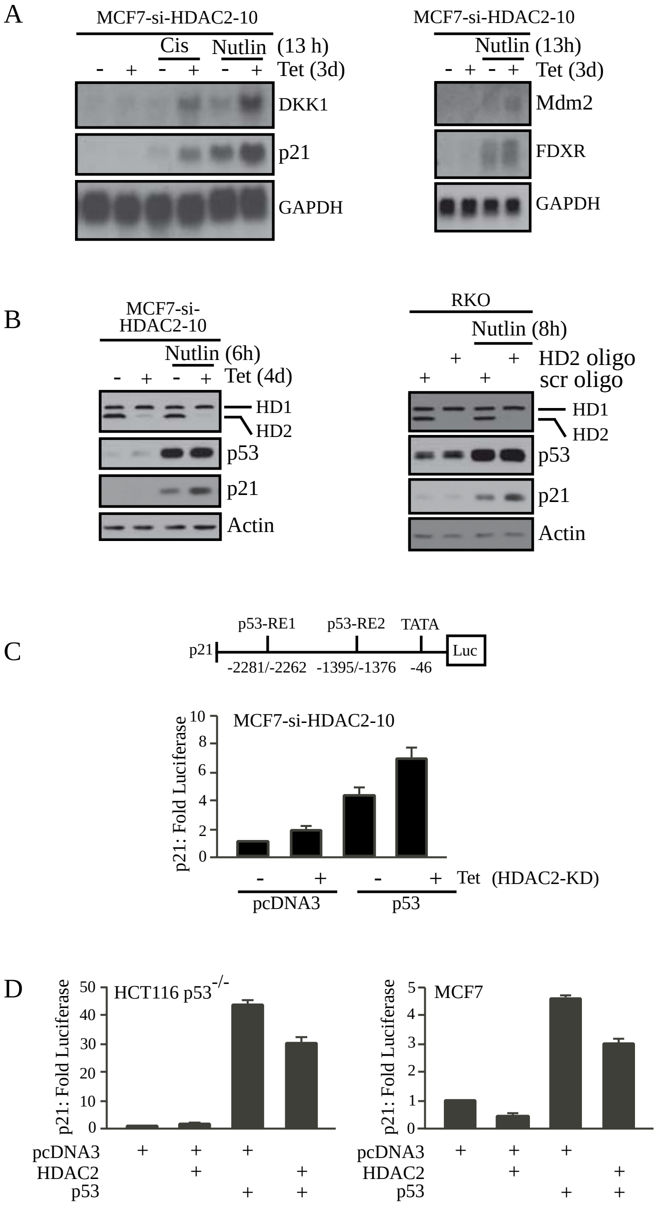
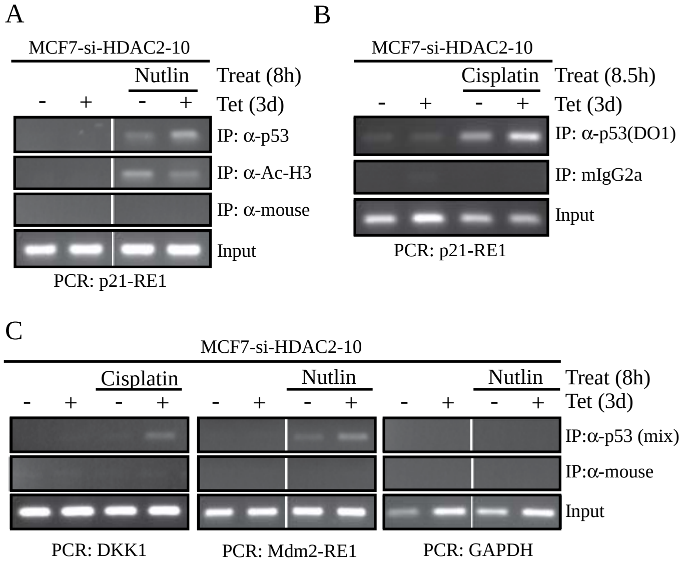
IP: rIgG
Input
c-Myc

Harms and Chen - Figure 5

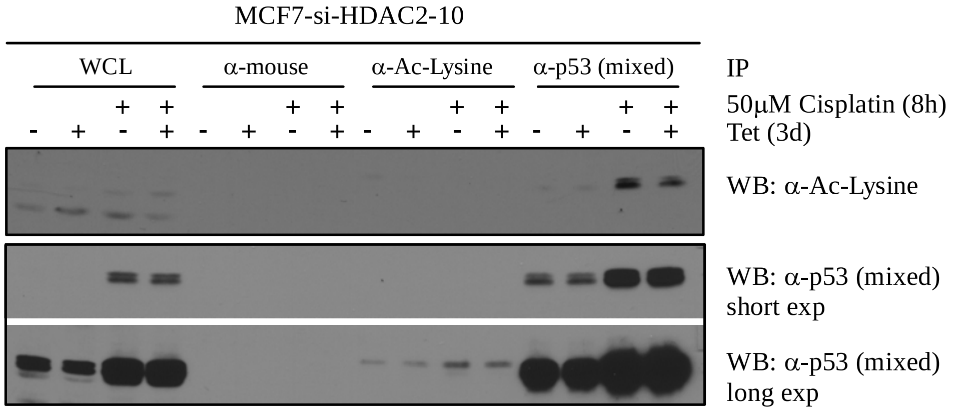


Harms and Chen - Figure 6



A
MCF7-si-HDAC2-10
Nutlin
Treat (8h)
- + - +
Tet (3d)
IP: α-p53
IP: α-Ac-H3
IP: α-mouse
Input
PCR: p21-RE1
B
MCF7-si-HDAC2-10
Cisplatin
Treat (8.5h)
- + - +
Tet (3d)
IP: α-p53(DO1)
IP: mIgG2a
Input
PCR: p21-RE1
C
MCF7-si-HDAC2-10
Cisplatin
Nutlin
Nutlin
Treat (8h)
- + - + - + - + - + - +
Tet (3d)
IP:α-p53 (mix)
IP:α-mouse
Input
PCR: DKK1
PCR: Mdm2-RE1
PCR: GAPDH

Harms and Chen – Supplementary Figure 1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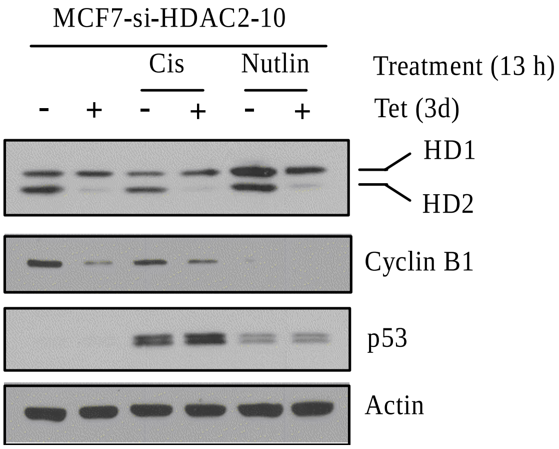
WCL
α-mouse
α-Ac-Lysine
α-p53 (mixed)
IP
+ + + + + + + +
50μM Cisplatin (8h)
- + - + - + - + - + - + - + - +
Tet (3d)
WB: α-Ac-Lysine
WB: α-p53 (mixed)
short exp
WB: α-p53 (mixed)
long exp

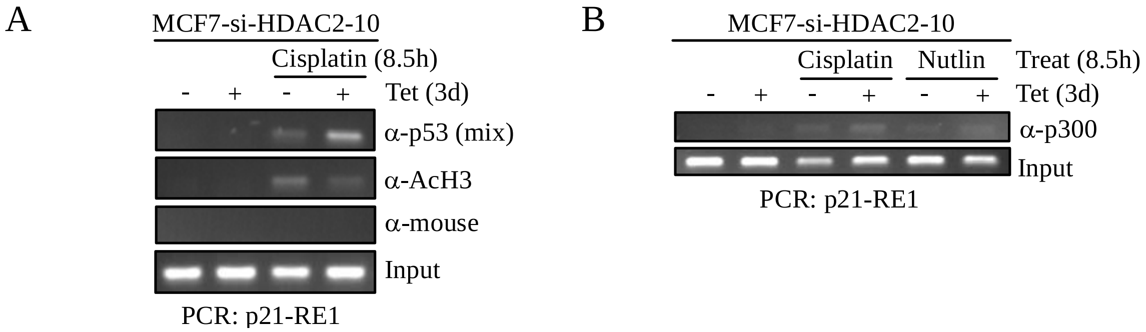
Supplementary Figure 1. Knockdown of HDAC2 does not augment cisplatin-induced acetylation of p53. MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3 days and treated with 50μM Cisplatin as indicated. Cell lysates were immunoprecipitated with anti-acetyl-lysine antibody (Upstate), anti-p53 antibodies (a mixture of DO1, PAb-1801, PAb-240, and PAb-421), or anti-mouse antibody (Sigma). The proteins in the immunoprecipitates were then sequentially detected with anti-acetyl-lysine antibody (Upstate) and anti-p53 antibodies.

Harms and Chen – Supplementary Figure 2


MCF7-si-HDAC2-10
Cis
Nutlin
Treatment (13 h)
- + - + - +
Tet (3d)
HD1
HD2
Cyclin B1
p53
Actin

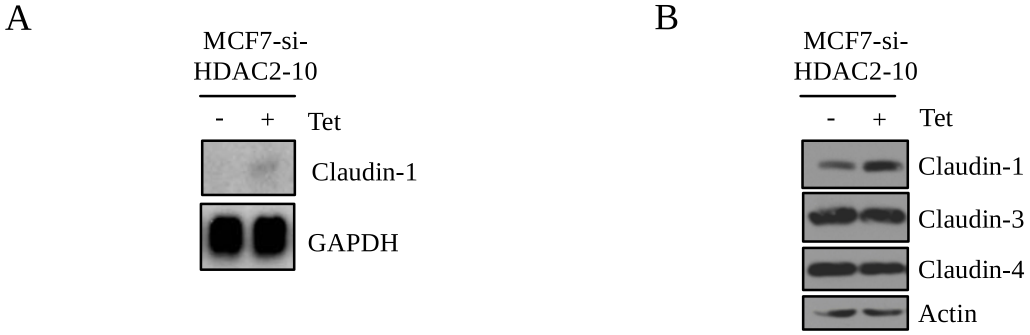
Supplementary Figure 2. Cyclin B1 is repressed by p53 and upon knockdown of HDAC2. MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3d and treated with 50 µM cisplatin or 5 µM nutlin for 13h. The level of HDAC1 (Upstate), HDAC2 (Upstate), Cyclin B1 (Santa Cruz), p53, and actin (Sigma) was assayed by Western blot analysis.

Harms and Chen – Supplementary Figure 3


A
MCF7-si-HDAC2-10
Cisplatin (8.5h)
- + - + Tet (3d)
α-p53 (mix)
α-AcH3
α-mouse
Input
PCR: p21-RE1
B
MCF7-si-HDAC2-10
Cisplatin
Nutlin
Treat (8.5h)
- + - + - + Tet (3d)
α-p300
Input
PCR: p21-RE1

Supplementary Figure 3. Knockdown of HDAC2 augments p53- and p300- binding and diminishes acetylation of histone H3 at the p21 promoter. (A) ChIP assay was performed using MCF7-si-HDAC2-10 cells grown with or without tetracycline and treated with 50 μM Cisplatin as indicated. Chromatin was immunoprecipitated with anti-p53 (mix of DO-1, PAb-1801, PAb-240, and PAb-421), anti-acetylated histone H3, or control anti-mouse antibodies. p53-RE1 within the p21 gene was amplified by PCR. (B) ChIP assay was performed using MCF7-si-HDAC2-10 cells treated as indicated. Chromatin was immunoprecipitated with anti-p300 antibodies. p53-RE1 within the p21 gene was amplified by PCR.

Harms and Chen – Supplementary Figure 4


A
MCF7-si-
HDAC2-10
- + Tet
Claudin-1
GAPDH
B
MCF7-si-
HDAC2-10
- + Tet
Claudin-1
Claudin-3
Claudin-4
Actin

Supplementary Figure 4. Knockdown of HDAC2 induces *Claudin-1,* but not *Claudin-3* or *Claudin-4*. (A) A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d. The blot was sequentially probed with cDNAs derived from *Claudin-1* and *GAPDH* genes. (B) The level of claudin-1, claudin-3, claudin-4, and actin was assayed by Western blot analysis with antibodies against claudin-1 (Zymed Laboratories Inc.), claudin-3 (Zymed Laboratories Inc.), claudin-4 (Zymed Laboratories Inc.), and actin in MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3d.